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Roy Waldo Miner

TISSUE CULTURE TECHNIQUE IN PHARMACOLOGY

BY

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November 17, 1954

Editor

ROY WALDO MINER

TISSUE CULTURE TECHNIQUE IN PHARMACOLOGY*

Conference Co-chairmen: C. M. POMERAT and ELMER L. SEVRINGHAUS
Consulting Editor: C. M. POMERAT

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* This series of papers is the result of a conference on *Tissue Culture Technique in Pharmacology* held by the Section of Biology of The New York Academy of Sciences, and The Tissue Culture Association, Duke University, January 14, 15, and 16, 1954.

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TISSUE CULTURE: PAST, PRESENT, AND FUTURE

By M. J. Kopac*

Department of Biology, Washington Square College of Arts and Science, New York University, New York, N. Y.

Ross Granville Harrison, in 1907, described his new method for studying the development of nerve fibers.[1] Whole medullary tubes, or fragments of them taken from frog embryos were mounted in a hanging drop of lymph fluid freshly drawn from lymph sacs of adult frogs. The tissue fragments became secured in the lymph, which quickly formed a clot. Such preparations, if aseptic precautions were taken, survived for a week or so, and could be studied conveniently under high magnifications of the microscope. One could see that the development of nerve fibers involved the outflow of protoplasm from central cells, and that this development was brought about, as Harrison pointed out, by "one of the very primitive properties of living protoplasm, amoeboid movement". The science and art of contemporary tissue culture was thus launched.

When Harrison's appointment to the Bronson Professorship in Comparative Anatomy in Yale was announced,[2] it was said of his work: "His latest studies on the histogenesis of living, isolated elements of tissues open up a new and fascinating field." History has adequately confirmed this statement, as evidenced by the recent publication of *An Index to the Literature of the Living Cell Cultivated* in Vitro, a bibliography representing about 15,000 articles.[3]

Since Harrison's pioneer experiments, there has been much progress. We have seen, for example, the development of Carrel flask techniques,[4] of roller tube techniques,[5] of perforated cellophane techniques,[6] as well as of replicate mass cultures.[7]

One should note the development of the single cell isolation technique for establishing pure-line clones.[8] This method is one of the truly important innovations for the study of homogeneous populations of cells. Until more highly refined procedures are developed, much of our biochemical knowledge can be obtained only from determinations made on large populations of cells. With a greater homogeneity of cells, the biochemist will be more reasonably justified in extrapolating the properties of a mass of cells to the single cell.

The mass replicate cultures with cells growing in fluid media, as well as the perforated cellophane preparations, provide cells as useful to the micrurgist as are the amoebas. The micrurgy of individual cells in plasma clot preparations, so extensively studied by Chambers[9] and others, was seriously handicapped owing to the annoying physical properties of the clot. With cells growing in a fluid culture medium, it is no longer difficult to conduct a wide range of micrurgical experiments. The recent studies by Duryee[10] are of significance, and they indicate a future extension of tissue culture to experimental cytology. A fund of important biological information can be harvested by transplanting subcellular structures from one cell to another. The potentials of this approach will be even more assured if the treated cells are kept alive long enough so that the effects of the subcellular transplants can be properly identified and evaluated.

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* Chairman, Section of Biology.

The value of tissue culture to the study of viruses is without parallel. It is well known that certain cells are more susceptible than others to the action of some viruses. Thin sections of such cells can be prepared and studied with the electron microscope. In many instances, the development of the virus, generally at the expense of some part of the cell, may be observed.

The future of tissue culture is even more promising as new horizons expand. To mention a few recent developments, there are: the development and availability, in 1954, of the Land ultraviolet color-translating microscope; the use of cultured cells for submicroenzymic cytochemical determinations;[11] the study of cytoplasmic inclusions by micrurgical transplantation, the development of ultraviolet micrurgy;[12] and the study of variability and stability of chromosome numbers in somatic cells.[13]

The conference on which this Monograph is based, sponsored jointly by The New York Academy of Sciences and The Tissue Culture Association, was organized to explore the utilization of tissue culture in pharmacology. On the one hand, tissue culture has much to offer to the study of drug action; on the other hand, it also has its weaknesses and pitfalls. Perhaps the most serious handicap, until proper methods can be developed, is the fact that tissue culture is not readily amenable to quantitative determinations.

This factor may be a blessing in disguise. The cell has become the frequent victim of statistics. It might be wise to point out that Kosolapoff,[14] in a short note, stated: "The statistical approach fails utterly to supply us with the basic, fundamental reasons for the events that do take place. . . . In addition, the statistical approach tends to close the door upon a closer, more intimate, and more individualistic approach to the basic understanding of the reasons for the collective behavior of a mass of units."

Although Kosolapoff's remarks were aimed primarily at physical phenomena, unquestionably such remarks could be directed even more profitably toward the study of living cells. What greater contribution could there be to the study of the mechanisms of drug action than to pinpoint with precision the part of a cell that may become altered by the drug? This qualitative approach, it seems to me, is more fruitful than the knowledge, for example, that 50 per cent of a population of cells is destroyed by a given concentration of a drug. What is it that is damaged or destroyed? What are the effects on cell division, on growth, or on motility? Perhaps, with this information, we may be able to show *how* and *why* such destruction or change occurs. As a result, we should know something new about drug action and, even more important, such observations may give us new clues regarding the function of subcellular structures in living cells.

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Part I. Pure Strain, Long Term and Mass Culture Methods. Commercial and Synthetic Media. Virology

RESPONSES OF A VARIETY OF NORMAL AND MALIGNANT CELLS TO CONTINUOUS CULTIVATION, AND SOME PRACTICAL APPLICATIONS OF THESE RESPONSES TO PROBLEMS IN THE BIOLOGY OF DISEASE*

By George O. Gey, Frederik B. Bang, and Margaret K. Gey
The Johns Hopkins Medical Institutions, Baltimore, Md.

For about half a century now, published reports show that investigators have progressively widened their interests in, and have more seriously examined the behavior of, explanted living tissues and their constituent cells, and that these investigators have tried in a number of ways to influence and control cellular activity. These biopsy studies of primary explants and continuous cell cultures have described work on tissues, especially from avian and mammalian hosts, in various stages of development up to senescence and death, and from experimentally altered and diseased hosts. Especially during the latter half of this period, there has developed an expanding interest in improving the techniques of tissue culture and a more serious application and planned projection of these methods to certain problems in the biology and control of disease. We have tried to contribute to these areas during this period.[1, 2, 3, 4, 5]

The technical achievements to date, and their potential applications, demonstrate the feasibility of comparing the derived normal or controlled state with a diseased state of the tissues or cells examined freshly during several days or more following biopsy, or as a more or less stabilized cell population during continuous cultivation. The tissue and cell responses *in vitro*, however, should be expected to vary somewhat from the natural state following varying periods of maintenance *in vitro*. In fact, whatever may have been the original state of the tissue prior to explantation, and however dependent, of course, it may have been upon the conditions imposed, one should be prepared to expect a variety of responses following explantation, including considerable shifts in cell composition. The responses of certain cells to continuous cultivation might be: a continuation of a previous rapid growth, as in a tumor; a continuation of an original dormant state (an apparently difficult state to maintain *in vitro*); a relative reversal of a previous state of increased physiological activity; a new transformation or adaptation;[6, 7] or, perhaps, whether wanted or unwanted, varying degrees of cell degeneration and death. In this latter category, we must hold ourselves responsible for the degenerative changes that have set in and attempt to account for them in a true spirit of scientific endeavor. So-called "nonspecific" degeneration, for example, often plagues those wishing to keep cells in a good state of health during continuous cultivation. Are the degenerative changes specific? Are they attributable to abnormal nutritional conditions, to the existence of microbial infection, or are they a kind of "end of the road" product, the so-called genetic lethals? Fortunately, we have learned, in some instances, that these degenerative changes can be accounted for on reasonable grounds. Nutritional abnormalities, especially those imposed by the use of heterologous media, are difficult to identify, but the effects are discernible,[8, 9] and reliable information can be obtained in this category.

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* These investigations were supported in part by a grant from the National Cancer Institute, U.S. Public Health Service, in part by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, in part by a grant from the National Foundation for Infantile Paralysis, and in part by the Medical Research and Development Division, Office of The Surgeon General, Department of the U.S. Army.

The media currently used for much tissue cultivation today is derived from animal sources and cannot easily be prepared free of viruses,[3, 4] and, at times, the effects of contaminating viruses may become apparent. In the electron microscope image seen in FIGURE 1, we see an illustration of parts of a cell presumably destroyed by particles indistinguishable from virus, yet, from an experiment where no deliberate addition of virus was made. Where an homol-

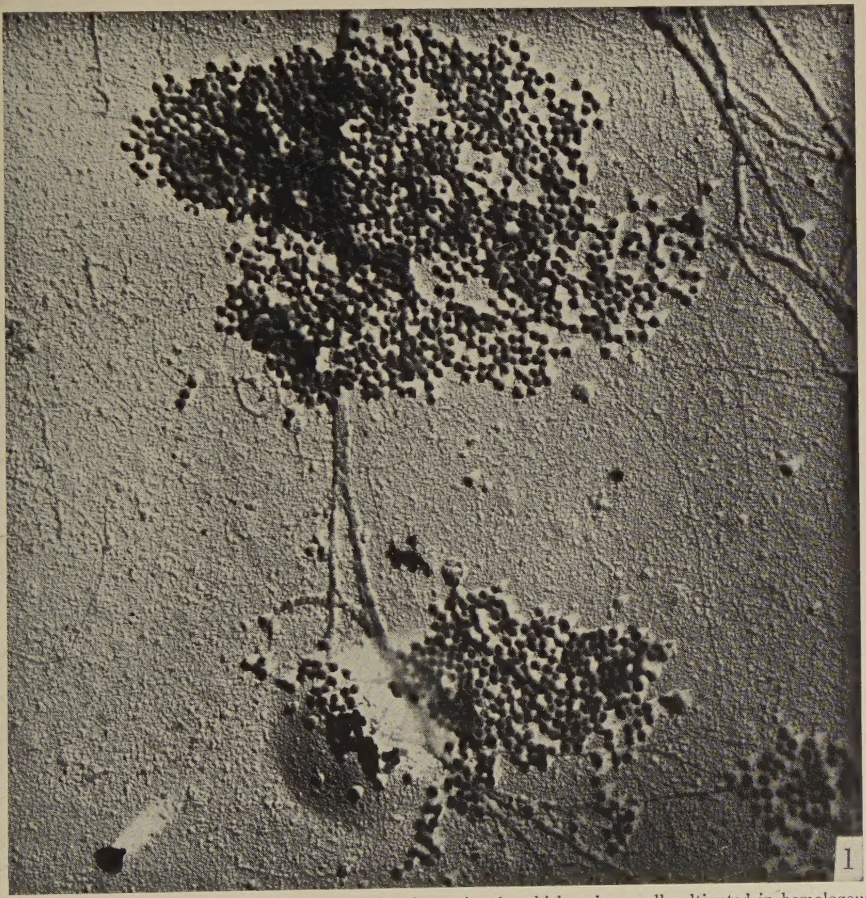
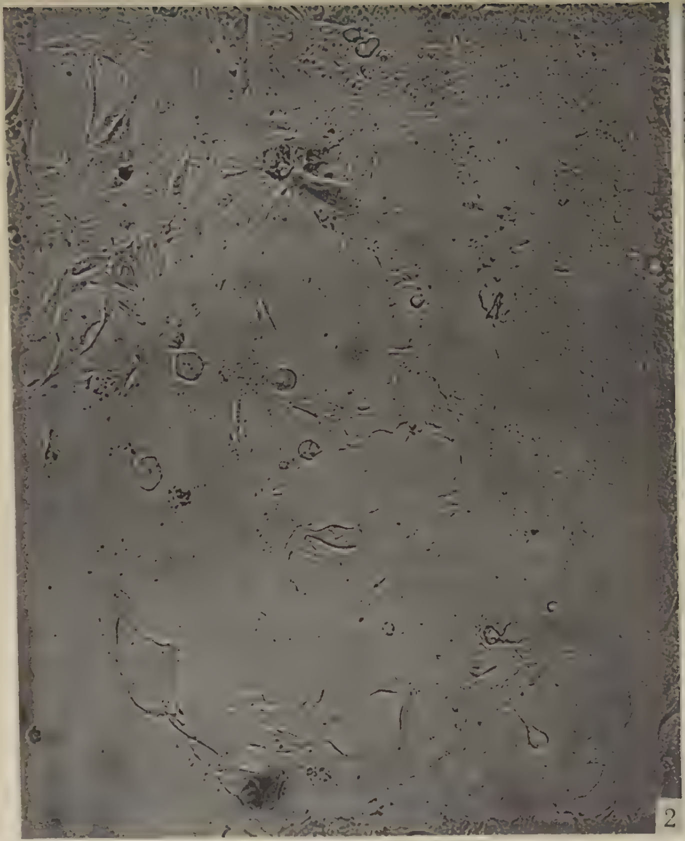


FIGURE 1. This illustration shows the peripheral margin of a chick-embryo cell cultivated in homologous stock media. Masses of particles indistinguishable from viruses are seen. Osmic acid fixation. Chromium shadowed specimen. Electron micrograph. Magnification ×15,000.

ogous set of conditions prevails, as in this example of chick cells grown in chicken media with plasma, serum, and embryonic extract, the effects of viruses known to parasitize chicken flocks must be realized as a real and potential hazard to the successful maintenance of continuous cell cultures derived from, or in the media of, this species. For in this day and age, our flocks, especially those of commercial origin, are known to have chronic enzootic virus infections. Difficulties of this sort would appear to jeopardize the maintenance of stable normal strains of cells grown in homologous media to a greater extent than many of the more rapidly growing tumor cell strains. Stable strains of tumor cells have been shown to support virus growth for long periods of time (FIGURE 2),[3, 4, 5] even in the presence of considerable evidence of cell destruction. The use of stable cell strains permits us to study directly the susceptibility of a variety of animal cells to a broad spectrum of viruses. We have, in fact, demonstrated extremes of susceptibility among normal and malignant cells of at least one species, the rat, when exposed to the virus of Eastern equine encephalomyelitis (EEE). See FIGURES 2, 3, and 4. The conditions of maintenance in fluid media with the cells grown directly on the glass wall in roller tubes permit us to evaluate, directly at the cellular level, the factors which alter susceptibility to virus.[10] Cultures which are known to be susceptible to virus and may show heavy destruction by virus may be seen to recover after prolonged cultivation with a complete restoration of grossly healthy colonies. This recovery is a good example of the ability of cells to survive in the presence of abundant virus. Such observations were made and reported on a series of rat normal and tumorous cells.[3, 4, 5]

More recently, in the case of the human HeLa cell strain of epidermoid carcinoma of the cervix, we have encountered a similar situation with early destruction after exposure to EEE, followed by recovery after prolonged cultivation. Here the initial destruction as seen in FIGURES 2 and 3 was rapidly followed by survival of colonies in good condition and showing a persistence of virus for long periods, even through repeated subcultures. In this strain, the evidence of the presence of virus is readily discernible, since the cells are usually thinly spread out. The surviving healthy population shows sufficient resistance to be able to phagocytize the virus-killed and virus-injured cells, and, in many cases, even the dividing cells may be shown to contain phagocyted cell debris. These recovery phenomena speak for cellular factors as playing an important role in this evidence of resistance to virus, since here no specific antibody has been deliberately introduced. In the earlier studies of several workers on vaccinia[11, 12, 13] virus in tissue cultures from immune rabbits, the virus was found to persist in cultures of immune corneas and of immune leukocytes. All of this work points to the importance of a further evaluation of these immune phenomena at the cellular level.

Many of the continuous cultures of various cell strains used today are grown in heterologous media. These cultures may show greatly altered cytological and growth responses or no grossly detectable change as compared to responses in strictly homologous media. We must extend our knowledge of the conditions which bring about varying degrees of cell degeneration and death of so-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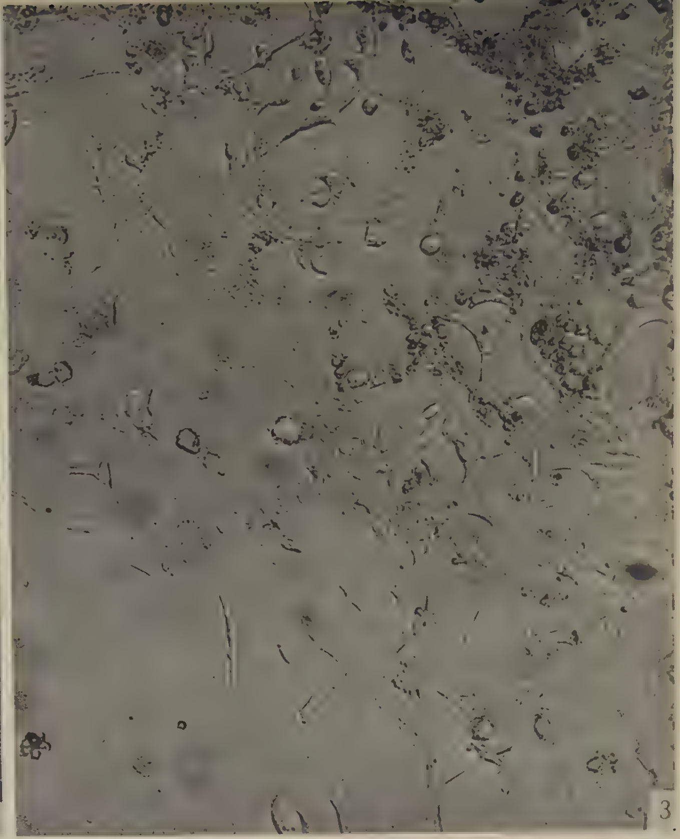
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Figure 2 shows a portion of a living roller tube colony of strain HeLa with fairly clear cells well spread out and with numerous dividing cells. Cultured 1,200 days in vitro. Magnification ×130.

Figure 3 shows a similar colony from an experimental tube exposed six days previously to one drop of EEE virus suspension (10^{-3}). Numerous degenerating and dead cells are still present and many have already washed away. The remaining cells are fairly clear and well spread out and include many actively dividing cells. Such active colonies, even through serial transfers, produce virus for prolonged periods. Magnification ×130.

called control cultures, if we are to understand the great variations and responses that occur when known virus infectious material is added.

The present expanding interest in the cultivation of animal viruses has led to repeated efforts to demonstrate the cytopathogenic effects of a variety of etiologic agents such as Far East hemorrhagic fever (FEHF) and homologous serum jaundice. The etiologic agents of these two poorly understood diseases, and perhaps many others, have yet to be isolated and grown in continuous culture. They represent reasonable examples of practical problems of this day, and one of them (FEHF) is referred to here, because we recently carried out a wishful episode of planned carnage on some of our helpless cultivated cells without getting the rewards we were seeking; *i.e.*, evidence of cell destruction in cultures maintained in the presence of virus. We refer particularly to an effort to induce cytopathogenic effects in cultured strains of human and rodent normal and malignant cells in a series of experiments designed to isolate the agent of Far East hemorrhagic fever in a field study.* The present failures of a number of workers to isolate by tissue culture means the etiologic agents of homologous serum jaundice, as in the current work of Bang *et al.* and of other groups, and of Far East hemorrhagic fever by Gey *et al.*, emphasize the need for a more precise use of the techniques of tissue culture. The extension of these techniques into categories more specifically concerned with providing a better understanding of the media factors affecting virus growth on or in cells, and of other factors concerned with the mechanism of cellular immunity and antibody production at the level of the living cell's response to virus, cannot be overemphasized.

During the early part of 1953, in connection with these hemorrhagic fever studies, we tried to increase the effective contact of viruses on cultivated cells by centrifuging the infected materials in glass tubes containing whole harvested tissue culture colonies. Under these conditions the cultured cell mass sediments first and, later, under the conditions of high gravity centrifugation, any virus material that may be present is deposited directly on the cells. Preliminary studies have shown that the cells tolerate relatively high centrifugal forces. It would be interesting to know how much stratification occurs in these cells as compared to other cell forms at lower gravity.[14] As a result of a series of experiments we now have good evidence of the effectiveness of this procedure, which is illustrated in TABLE 1. Here it may be seen that two human tumor cell strains, A.Fi. and HeLa, will tolerate three hours' exposure to forces operating at over 100,000 times gravity, even at temperatures approximating the body temperature (36.5° C). The medium used in these experiments was 20 per cent human placental cord serum. In almost every single instance a very good tissue culture recovery was obtained from the sedimented cell mass. In the figure shown on FIGURES 6 and 7 a control culture of the 15-year-old human fibrosarcoma strain A.Fi. may be compared with the culture shown three days after an exposure to over 100,000 times gravity for three hours at 37° C. The culture which has been subjected to high gravity shows a few rounded, apparently degenerating cells, and some cell debris, whereas the control culture

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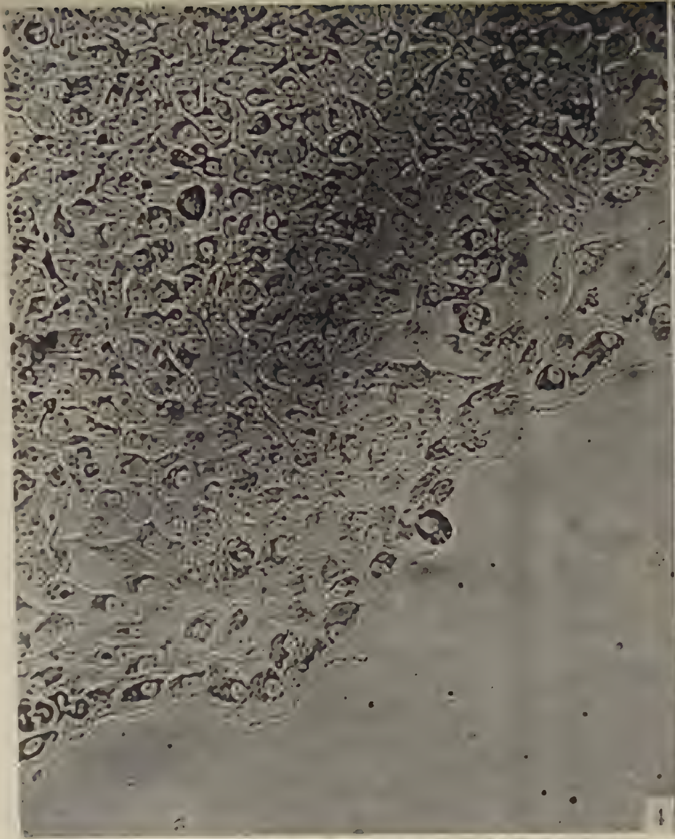
* These field studies in Japan and Korea (spring 1953) were conducted by Gey and Bang and were sponsored by the Commission on Hemorrhagic Fever of the Armed Forces Epidemiological Board, and supported by the Office of the Surgeon General, Department of the U.S. Army. To be published.



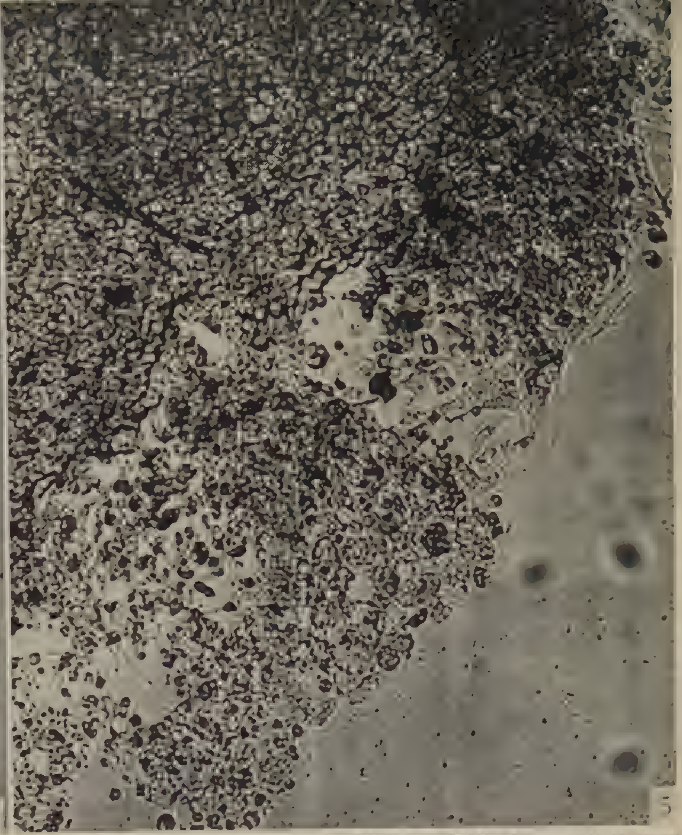


FIGURE 4 shows a portion of a living roller tube colony of the HeLa strain two days after one drop of EEE virus suspension (10^{-1}) was added and represents the experimental control for the ultracentrifuged colony shown in FIGURE 5. Note the early granular appearance of these noncentrifuged control cells as a result of two days' exposure to the virus. Magnification ×130.

FIGURE 5 shows heavy destruction of the colony two days after exposure to the same amount of fluid containing virus as used on the noncentrifuged experimental control (FIGURE 4) and after centrifugation (100,000 × g) for one hour at 37° C. It is apparent that more virus has been deposited and has produced more extensive and earlier destruction. A few well expanded viable cells are still present. These will eventually reform an intact colony but with a persistence of virus through serial transfers.

TABLE 1

RESPONSE OF VARIOUS STRAINS OF CELLS TO ULTRACENTRIFUGATION

(1953 experiments)

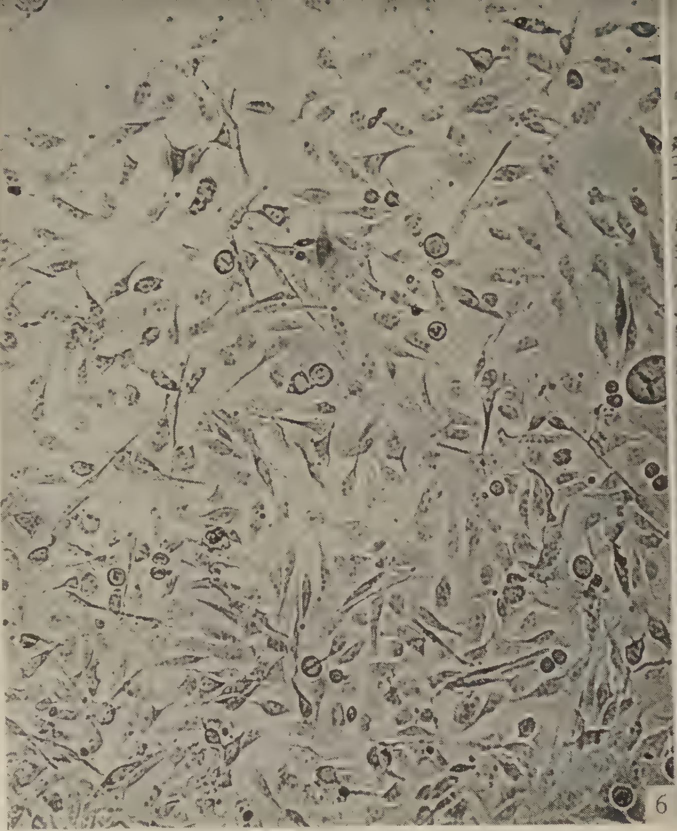
Exp. No.	Strain	Cord serum conc.	Temp. ± 1.0°C.	Time (hrs.)	R.C.F.	Tissue culture recovery
1	HeLa	10%	22	0.5	26,500	Very good
2	A.Fi.	20%	26–17	3	81,500	Very good
	14p	“	“	“	“	“ “
	72	“	“	“	“	Fair
3	A.Fi.	20%	36.5	3	81,500	Very good
	14p	“	“	“	“	“ “
	72	“	“	“	“	“ “
4	A.Fi.	20%	36.5	3	81,500	Very good
	HeLa	“	“	“	“	“ “
	14p	“	“	“	“	“ “
	72	“	“	“	“	“ “
5	A.Fi.	20%	36.5	3	111,000	Good
	HeLa	“	“	“	“	“
	14p	“	“	“	“	“
	72	“	“	“	“	“

The cell strains listed recovered completely in all the experiments. The “fair” recovery given for strain 72 Experiment 2, refers only to the early response a few days after centrifugation and was a temporary effect.

R.C.F. = Relative centrifugal force (gravities).
HeLa = Human epidermoid carcinoma of the cervix: 3 yrs. in culture.
A.Fi. = Human fibrosarcoma: +15 yrs. in culture.
14p = Rat fibroblast: +15 yrs. in culture.
72 = Rat fibrosarcoma (in vitro origin): +12 yrs. in culture.

shows very little evidence of degeneration or cell destruction. In both cases there are a number of dividing cells present. Further cultivation of such cultures, or of cultures of the HeLa strain exposed in the same manner, show excellent recovery on prolonged cultivation with persistence of virus.

Also, as may be noted in TABLE 1, a study was made of the normal rat fibroblast strain 14p and an autologous tumor cell line No. 72 of *in vitro* origin. Here again, these strains tolerate very well the prolonged exposure to high gravity. By this technique we have been able to sediment viruses directly and rapidly on living cells in an effort to demonstrate mass virus destruction in as short a period as possible. We are, in fact, convinced that this procedure can be applied to thinly spread out, intact colonies and thereby permit an evaluation of focal necrotic lesions[3] or plaques[15] under conditions of maximal virus exposure. In a series of experiments we now have evidence that viruses sedimented on tissue cultures cause destructive effects of greater magnitude in the centrifuged cultures as compared to the virus treated controls not centrifuged. When colonies of HeLa strain are subjected to one hour of centrifugation at over 100,000 × g. and exposed to EEE virus suspension (10^{-9}) during centrifugation, a heavy destruction can be seen in cultured colonies within two days as shown in FIGURE 5. The noncentrifuged experimental control cultures showed only a beginning granular appearance of the cells when exposed to the same amount of virus suspension in roller tube culture. The results of such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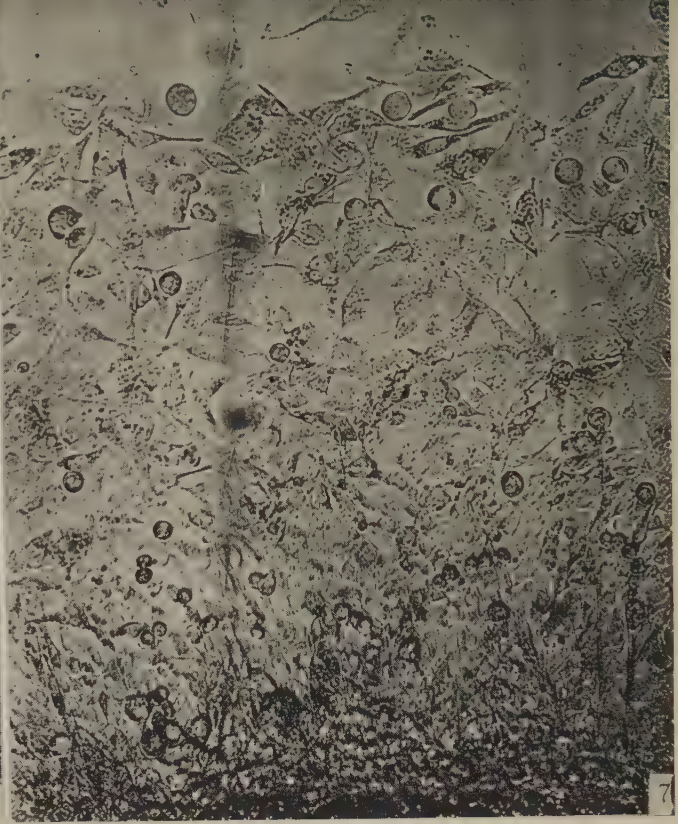
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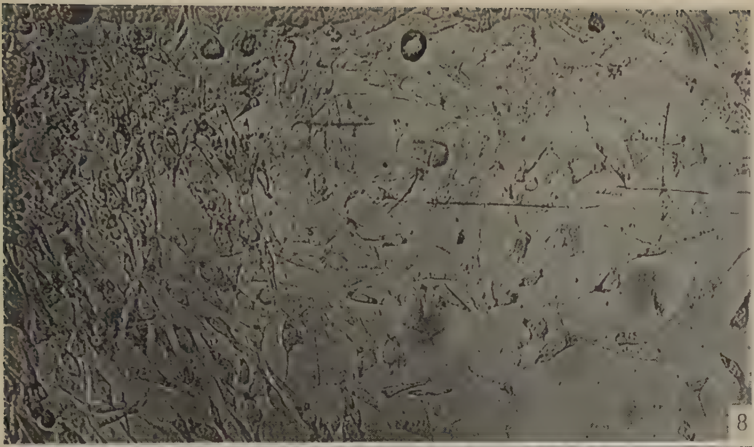
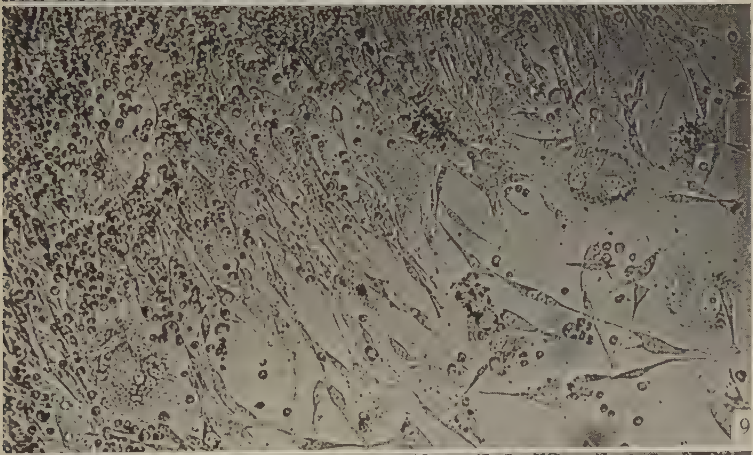
FIGURE 6 shows a control culture of the human fibrosarcoma strain A.Fi. The cells are well expanded and fairly clear. Many dividing cells are present. Living roller tube image of 15-year-old strain. Magnification ×130.

FIGURE 7 shows a comparable colony of the same strain grown three days after exposure to high gravity (100,000 × g.) for three hours at 37° C. The cells are slightly more granular than the control colonies; there are some degenerating cells as well as some cell debris. Many dividing cells are present. This condition rapidly clears up with prolonged cultivation. Living roller tube image of 15-year-old strain. Magnification ×130.

exposure to EEE virus suspension (10^{-9}) after two days' growth is seen in FIGURE 4.

The spectrum of cell types that may be grown indefinitely is already a large number, even when judged alone from the experiences of our laboratory. A few practical examples should illustrate how successfully, or unsuccessfully, we have plied our trade in the management of a host of explanted human and animal tissues, and to what extent these examples may help us in the study of various aspects of disease processes. For some studies it would seem desirable to isolate specific strains of cells, and to determine their stability and metabolic activities in fixed environmental conditions and in a somewhat controlled nutrient substrate. We have already emphasized the importance of the absolute sterility of such materials, especially for virus studies. Many cell strains established *in vitro* during this past half century have already demonstrated their stability, whether taken from embryonic or young or old hosts. The simplest of continuous culture methods would seem to offer and, in some cases, have already demonstrated the opportunities that are afforded for a study of differentiation, or perhaps reversal or loss, of some differentiated characteristics. In some cell strains most remarkable cellular variations have been observed, including even the transformation of normal cells into malignant ones[6, 16, 17] and, in some cases, the loss of transplantability of these tumorous lines.[18, 19] Loss of transplantability, in some cases with and in others without profound changes in cultural characteristics, have been demonstrated. For example, Earle's strain "L" tumorous mouse fibroblasts, our strain T-333 of tumorous rat fibroblasts, and de Bruyn's strain MB-III (lymphoblastic cells of tumorous origin) showed loss of transplantability. One of the most interesting of these transformations occurred in Doctor de Bruyn's cultures grown in this laboratory several years ago;[18] representatives of these original cultures are still maintained in this laboratory. FIGURE 9 illustrates a mixed culture of the highly malignant strain MB-III which retains its transplantability and vigorous tissue growth only in the presence of abundant fibroblasts. Two interesting situations, demonstrated in connection with transplantable tumors, have been recorded by de Bruyn. A stable tissue culture, as shown in FIGURE 9, may lose its malignant lymphoblastic components and may produce a fibroblastic strain, as shown in FIGURE 8. These cultures are incapable of producing tumors. Or a more remarkable change may occur with the production of "altered" vigorously growing lymphoblasts, as shown in FIGURE 10. These lymphoblasts do not produce a tumor. It must be kept in mind that these cells were derived from sick cultures which eventually lost their supporting fibroblasts and managed, through selection or adaptation, to produce eventually a vigorous growth. These MB-III transformed lymphoblasts no longer produce a tumor even when recombined with healthy fibroblasts. They can be grown as freely suspended cells in agitated fluid media.[20] These experiences in our laboratory help to emphasize the importance of understanding the factors which alter the cellular responses to cultivation. One might even postulate that a virus had wiped out fibroblasts and perhaps some of the more susceptible tumorous lymphoblasts. Doctor de Bruyn has stressed the importance of the use of heterologous media as a factor.





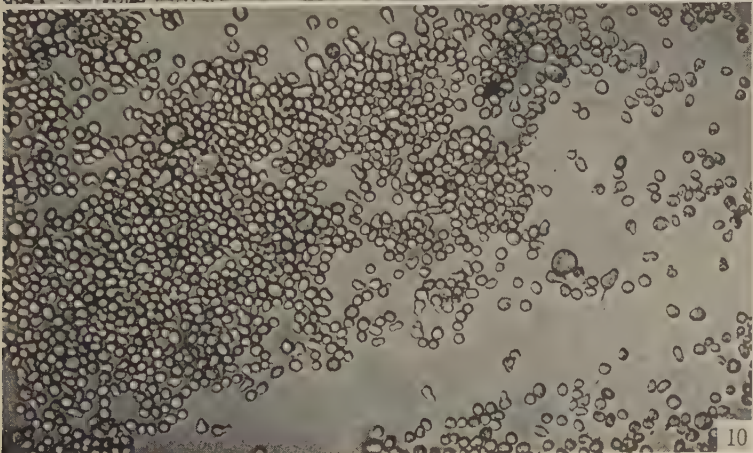


FIGURE 8 shows a living roller tube culture of mouse fibroblasts derived from the lymphoblastic tumor strain MB (de Bruyn). Cultured as fibroblasts for five and one-half years. These do not produce tumors. Magnification ×115.

FIGURE 9 shows the stable mixed culture of malignant lymphoblasts and fibroblasts cultured over 6 years in roller tubes. Such cultures consistently produce tumors. Living culture. Magnification ×115.

FIGURE 10 shows masses of living small and large round cells. Some are of the typical lymphocyte type with tails. Grown in fluid media in roller tubes on glass. This transformed strain, MB-III, cultured over 6 years in fluid media, has a rapid growth rate but does not produce a tumor. It can be grown as freely suspended cells in agitated fluid medium. Magnification ×115.

Also in the case of Earle's sarcoma "L" line, which has lost its transplantability, the specific factors leading to this change have not been revealed. Since variation in genetic susceptibility of the animals receiving the implants of cultured cells may play a role here, one must also consider the occurrence of altered susceptibility through natural mutation of the animal strains originally known to be susceptible to these various tissue culture strains.

In addition to the examples cited, our greatly altered rat-cell strain of tumorous origin T-333 showed, after many years of ready transplantability to rats from tissue culture, a loss of this growth potential; this loss was without any perceptible change in its rapid growth rate in tissue culture, in its manifest polyploidy, and in its rapid pinocytosis. Here again, one must consider the role of variation in the inbred rat strain from which the original cells were derived and which now may be resistant to transplantation. This strain, T-333, differs in many ways from its specific normal prototype 14p as described earlier.[6] Its high susceptibility to EEE virus is shown in FIGURES 11, 13, and 14. This result points to the need for more evidence on the comparative susceptibility to viruses of malignant cells and their specific normal prototypes. In fact, we have emphasized for many years the importance of creating and working with isogenic pairs of normal and malignant cells. We have tried on many occasions to create such strains from human tumors and from the specific

FIGURE 11 shows a 15-year-old strain, 14p, of adult rat mesenchyme cells of subcutaneous areolar tissue origin. These grow in fluid media as a single-cell layer on glass. This culture treated with undiluted EEE virus grown in the embryonated egg is apparently unaffected by virus. Culture looks like the control. Reprinted from 1951. See REFERENCE 3. Magnification ×130.

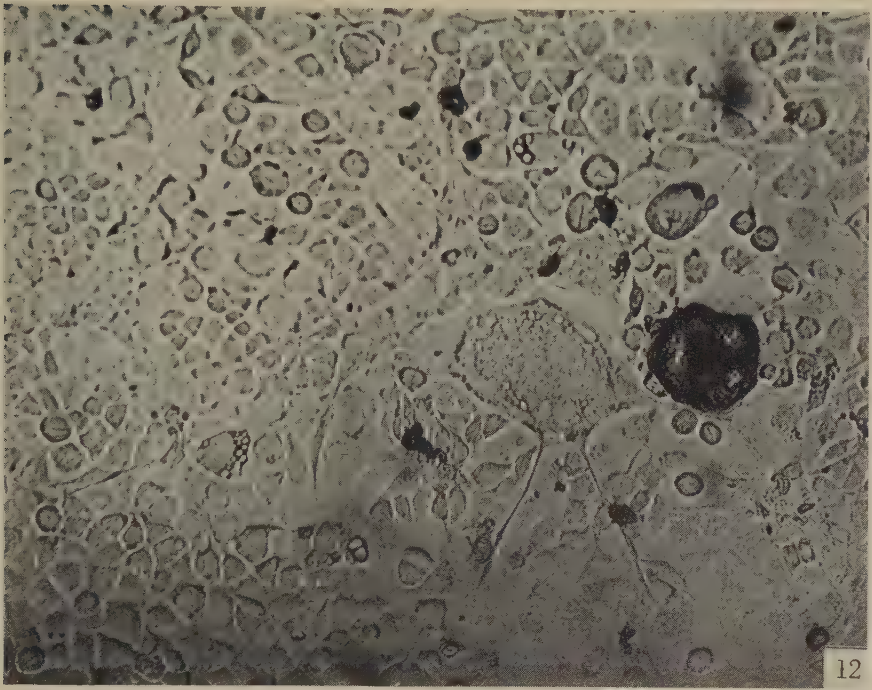


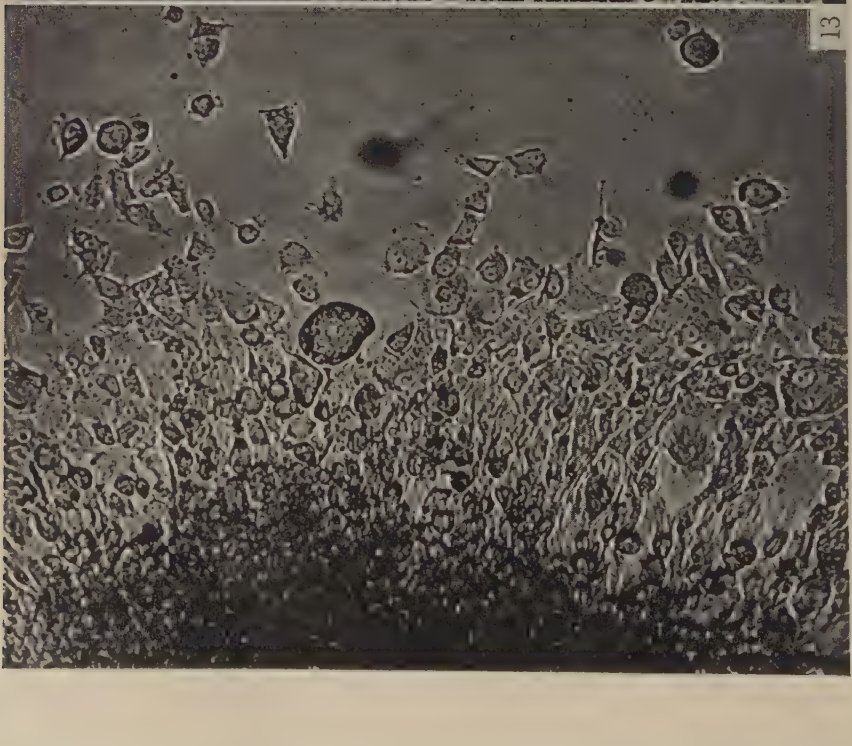
FIGURE 12 shows a 12 year-old strain of rat sarcoma 72 of *in vitro* origin. EEE virus grows in this strain for apparently indefinite periods with destruction of some cells. Note cell casts and the large dead multinucleated cell adjacent to a healthy one. Many dividing cells present. Reprinted from 1951. See REFERENCE 3. Magnification ×140.

normal tissue from which the tumors arose. The results of a few of these efforts will be described briefly.

Although we have had considerable experience with the growth of normal adult human fibroblasts established as stable strains[1] and have had an opportunity to compare their cultural characteristics with sarcomas from the same patient, at no time in our earlier work were we certain of their specific histogenic origin. More recently we have succeeded in establishing autologous human strains of normal and malignant cells of the same specific tissue origin. One of the more interesting of these strains was derived from a chondrosarcoma provided through the courtesy of Doctor W. M. Firor; later some normal rib cartilage was also provided from the same patient.

FIGURE 16 shows a section of this chondrosarcoma which was removed almost four years ago. A similarly stained section of a slowly growing tissue culture mass is shown in FIGURE 15 for comparison. It must be emphasized that although this tumor grew slowly in the patient and in the tissue culture, it nevertheless showed malignant extension and destroyed the adjacent tissues with an eventual extensive involvement and a fatal course.

An example of the character of a single tissue culture colony may be seen in FIGURE 17. This stable strain produces greatly elevated, thick colonies which



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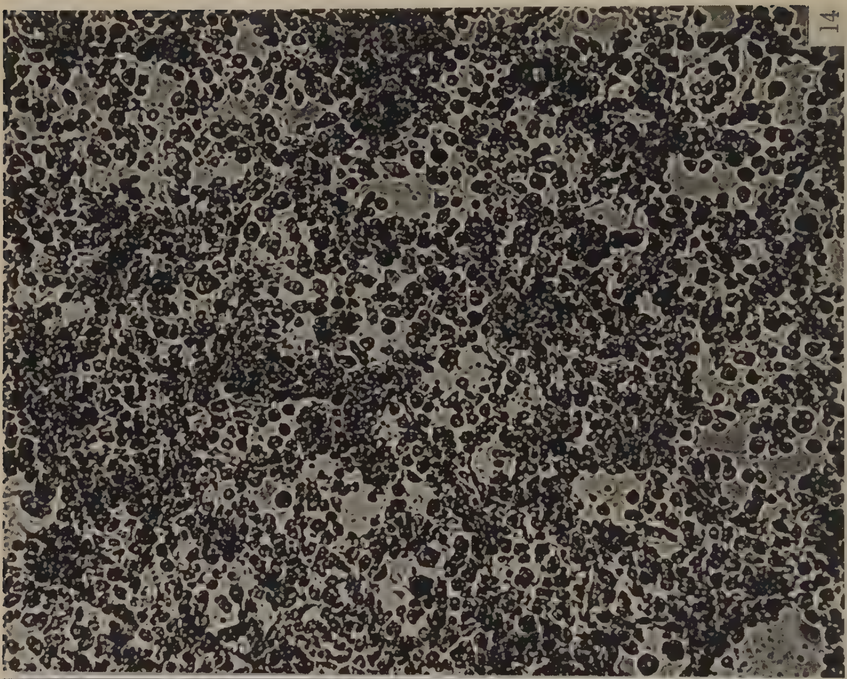

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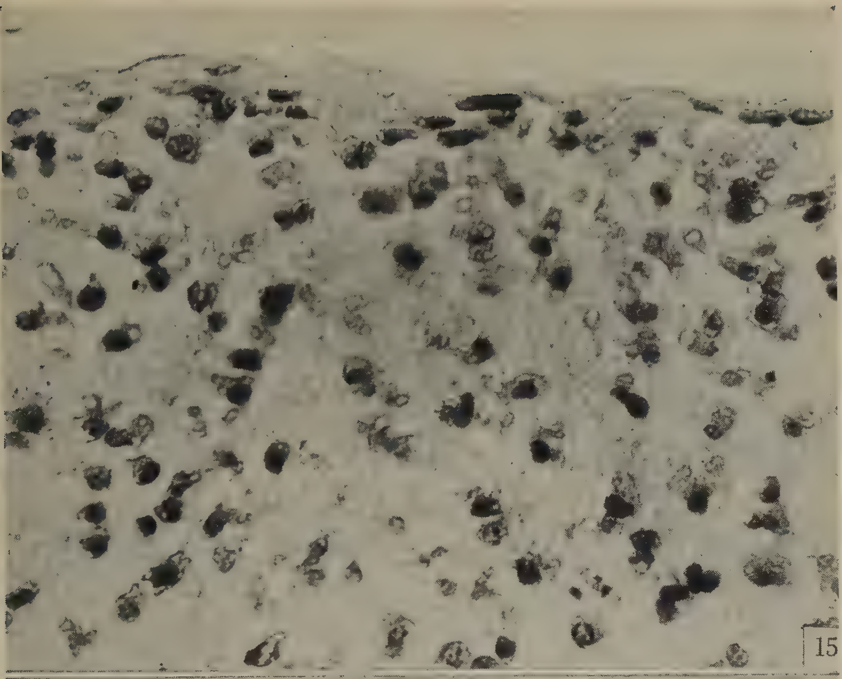
FIGURE 13 shows a control culture of 10-year-old strain of rat sarcoma strain T-333 derived in tissue culture from normal strain 14p. Cells grown in fluid media on glass. Tumor cells larger than normal prototype with much greater variation in cell size. Reprinted from 1951. See REFERENCE 3. Magnification ×130.

FIGURE 14 shows a culture of same strain, T-333, destroyed by EEE virus. Dark cell casts remain. Reprinted from 1951. See REFERENCE 3. Magnification ×130.



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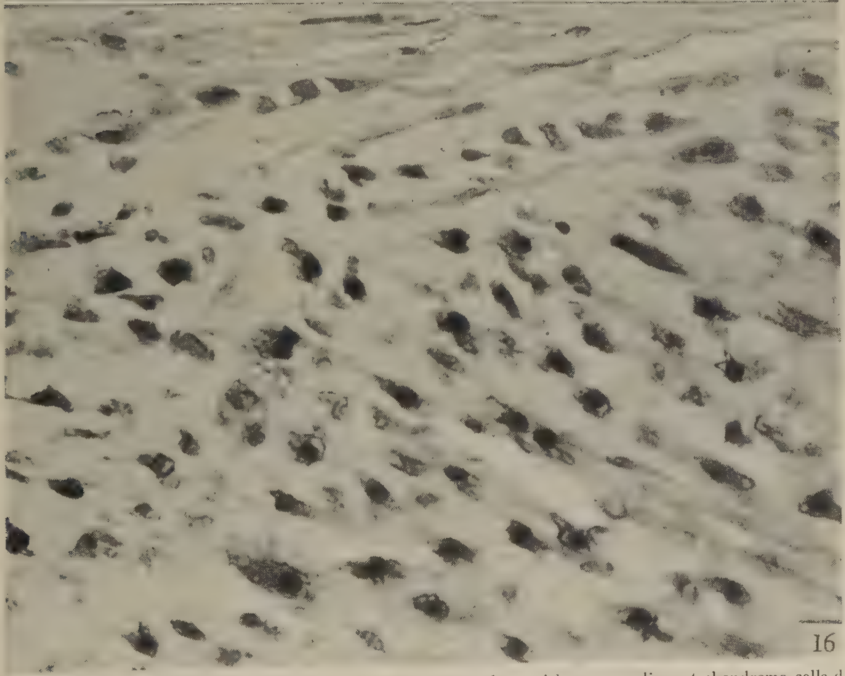

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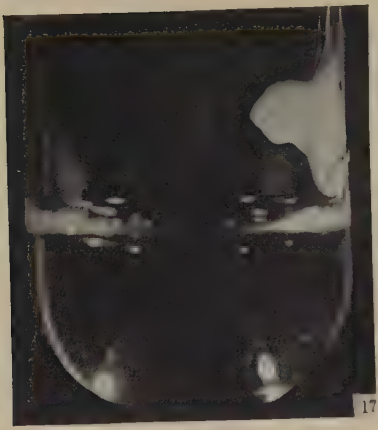
FIGURE 15 shows a histological section of a tissue culture colony with many malignant chondroma cells dispersed in abundant matrix. The cells in this culture remain enclosed and grow inside a firm capsular envelope. H and E strain. Magnification ×260.

FIGURE 16 shows a comparable group of cells in a section of the biopsy specimen. The cells are more abundant and have less matrix about them. Here they are enclosed in a fibrous capsule. H and E stain. Magnification ×260.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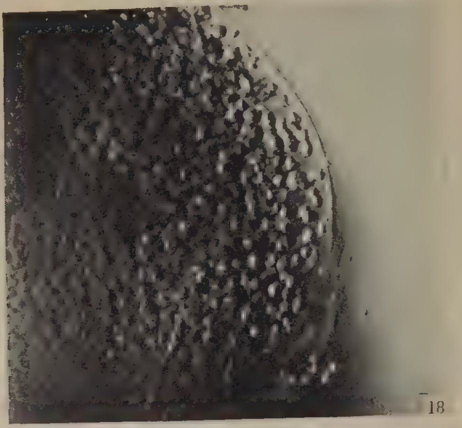
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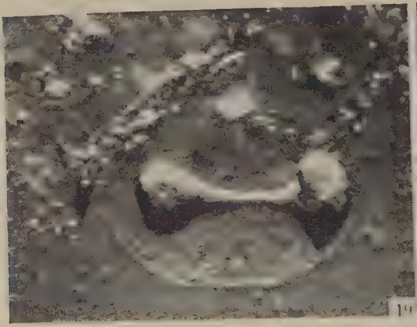
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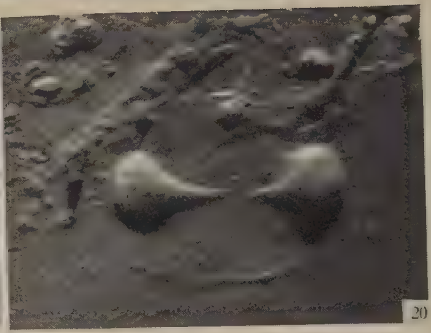
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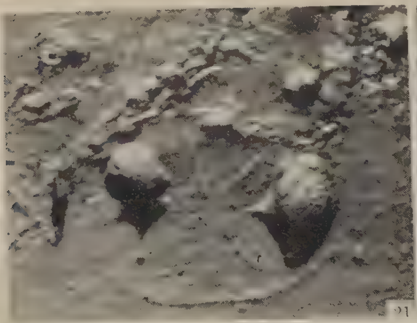
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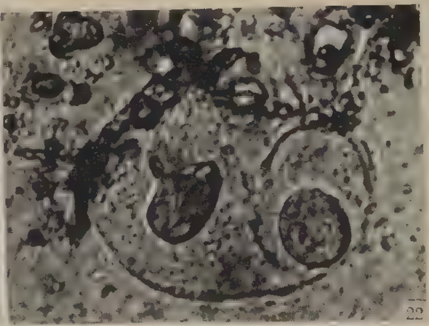
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FIGURE 17 shows a single living thick protruding JeRe chondrosarcoma colony in a roller tube culture. Cultured 300 days. Magnification ×2.8.

FIGURE 18 shows the transparent edge of a small spherical colony and reveals many irregular nuclei in the living state. The entire cell mass is enclosed in a capsular envelope. Cultured 112 days. Magnification ×90.

FIGURE 19 shows a living chondrosarcoma cell, strain JeRe, with a greatly elongated hourglass nucleus undergoing amitotic division in roller-tube culture. Note the thick cell membrane and faintly imaged short mitochondria. Magnification ×450.

FIGURE 20 shows the first evidence of nuclear separation four days later. Magnification ×450.

FIGURE 21 shows a more rounded condition of the daughter nuclei two weeks later and with deep cleavage furrows in the cell but not complete separation. Magnification ×450.

FIGURE 22 shows, at 36 days, a well rounded daughter nucleus and an almost completely separated daughter cell. The other daughter nucleus still shows a blunt telophase spike. Note the numerous short, thick mitochondria. The smaller daughter cell appears to be still enclosed by the parent cell membrane. Cultured 1,140 days. Magnification ×450.

in many ways resemble the growth of crown gall tumors of plants grown on agar. The appearance of the edge of a small colony is seen in FIGURE 18, which shows quite well the character of the capsular envelope. In our experience this is one of the most slowly growing strains of cells of a malignant variety that we have ever encountered. One example of some of the amitotic cleavages that are seen in this strain is illustrated in FIGURES 19 to 22. Here we see in FIGURE 19 a single living cartilage cell outside the main rounded tissue culture colony having a greatly elongated hourglass-shaped nuclear mass. In FIGURE 20 the two new masses have just separated during an interval of four days. In about two weeks a more complete separation is established as is seen in FIGURE 21. Thirty-six days following the condition found originally in FIGURE 19 the new daughter cells have almost completely separated, but they still lie within a common cell membrane. These remarkable living photographs of a single cell cleavage in a roller tube culture are but one example of the slow growth rate of this tumor.

We have maintained, during a comparable period of time, cultures derived from the autogenous rib cartilage. These originally were carried on in continuous cultures as discrete cartilagenous fragments surrounded by atypical fusiform fibroblasts, which now continue to grow very slowly as a strain but do not produce typical cartilage cells. This autogenous pair of cells—normal and malignant—provides excellent material for comparative study, but they are difficult to manage. Other attempts with new material may be more successful. We are greatly indebted to Mrs. Mary Toye Kubicek and to Doctor Maria Svotelis for carrying these strains through difficult periods of survival.

There has been a considerable growth in the application of tissue-culture techniques to the problem of virus cultivation and isolation. In some of these new activities, our stable strain of human epidermoid carcinoma of the cervix, strain HeLa, has been used. To date a large number of viruses has been isolated and propagated[21, 22, 23, 24] in cultures of this human strain, which is now widely distributed throughout the world. This strain, whose origin and proliferative capacity is about to be described briefly, is quite susceptible to the polio viruses; its response to mass culture procedures is now under study in several places. The 15-year-old fibrosarcoma strain, A.Fi., also susceptible to polio viruses[25] has also been distributed to a number of laboratories.

The HeLa strain is now over three years old, and a tissue culture recovery of a guinea pig passage (anterior chamber of the eye) appears to behave like the strain implanted originally. This is our oldest carcinoma strain.[26] It grows abundantly in a variety of media substrates, including fluid cultures, with cells grown directly on glass using only diluted (30 to 50 per cent in B.S.S.—Gey) placental cord or adult human serum, horse serum, or guinea pig serum as the fluid medium. Other combinations of synthetic media and biological fluids are producing good growth. Sometimes profound changes occur when these cells are maintained in such heterologous media (horse, guinea pig). As yet the nature of the changes is not clearly understood. FIGURE 23 shows the rounded granular condition of this strain in a diluted horse serum medium. Other samples, however, can be maintained in horse serum in good condition. Indeed,

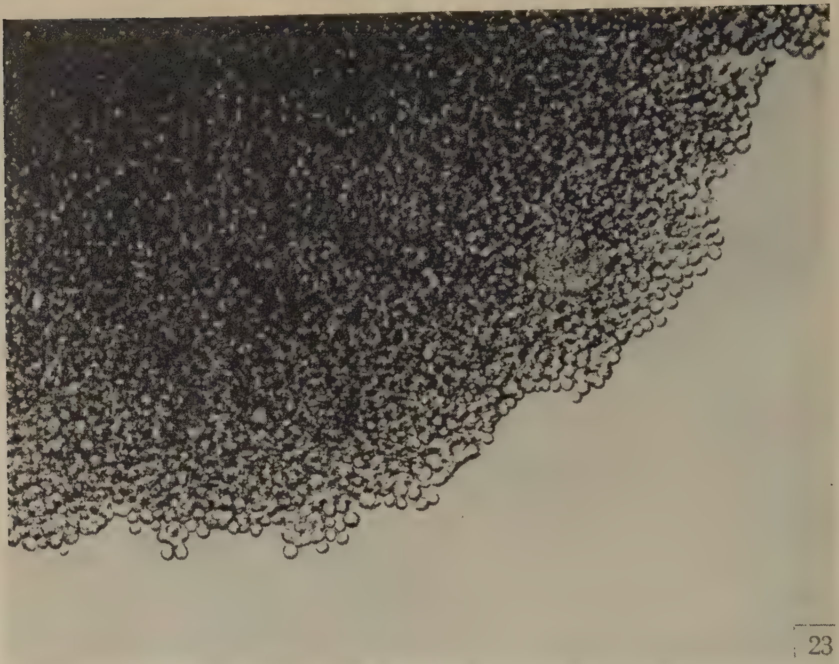
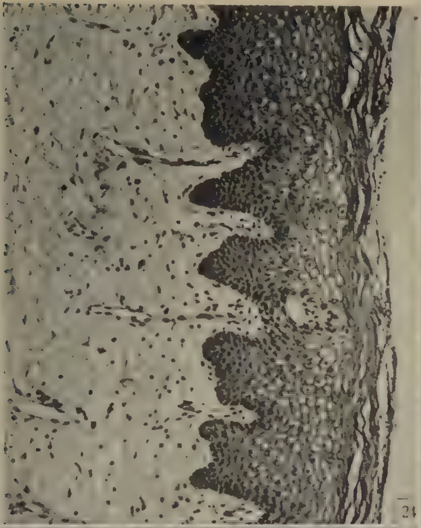


FIGURE 23 shows the rounded and granular character of HeLa cells in a thick irregular colony. Cultured in diluted horse serum plus mixture 199, of Morgan, Morton, and Parker, equal parts. This is an acquired condition which survives repeated transfers owing to the presence of virus or poor adaptation to this medium. Such cultures will recover in cord serum. Magnification ×130.

in some cases a "persistent virus" has been thought to account for the changes found during such prolonged cultivation in such media. The HeLa strain will also tolerate the violent action of continuous agitation when freely suspended cell cultures are maintained in agitated fluid medium. During such cultivation many colonies stick to the glass and are unaffected by the turbulent medium. In connection with the Far East studies we discovered the ability of these cells to manifest synchronously dividing cells following a period of cooling and subsequent reincubation. This important finding has led us into an evaluation of cellular variations in radiosensitivity correlated with some control of mitosis in malignant cell populations.

The autologous normal cervical epithelium shown in FIGURE 24, to be compared with its malignant derivative shown in FIGURE 25, was grown well over a year as a rapidly keratinizing cell strain, but actually it grew only about 5 per cent as rapidly as its malignant derivative. The normal cells of the slowly growing culture may be seen in FIGURE 26, and may be compared with the malignant HeLa cells of FIGURE 27. A postirradiation, autologous "normal" area yielded a slowly growing cell strain composed of small epithelial cells quite different from the malignant and the normal prototypes, thus indicating that the gamma radiation which had wiped out the malignant cells was not without a permanent effect upon the surviving normal cells.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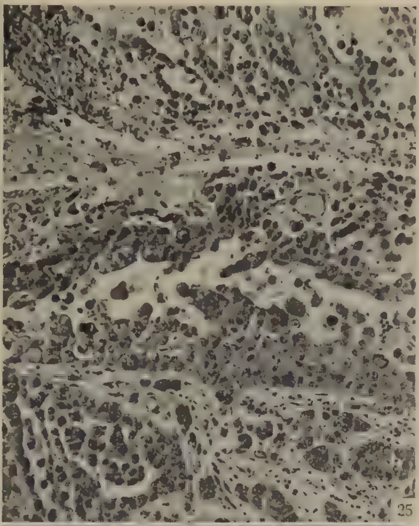
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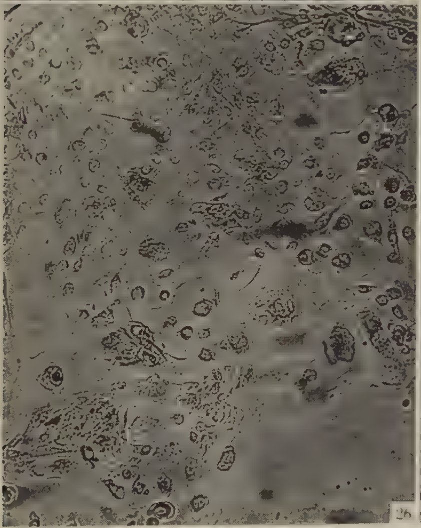
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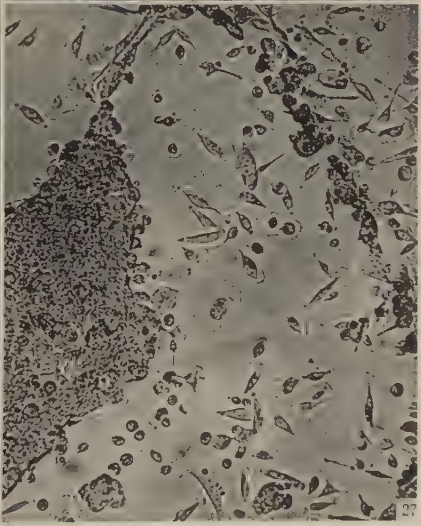
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FIGURE 24 shows an H and E stained section of the normal cervical epithelium biopsied from an uninvolved area opposite the HeLa lesion and may be compared to Figure 25 from a similarly prepared and stained section of the HeLa epidermoid carcinoma. Magnification ×100.

FIGURE 26 shows the slowly growing normal epithelial cells in a roller tube culture maintained through severa transfers for 97 days. This strain rapidly keratinized itself out of existence after over 400 days' cultivation. Living image. Magnification ×85.

FIGURE 27 shows a portion of a roller tube colony of the HeLa strain after 56 days' cultivation. Repeated transfers demonstrated its rapid growth. No prominently keratinized cells have been found in over three years' continuous cultivation. Living image. Magnification ×85.

We have repeatedly attempted to isolate vigorous strains of isogenic normal and malignant epithelial cells of the cervix from many other cases but have succeeded only in isolating slowly growing keratinizing cell lines, which are most difficult to maintain. An example of a nonkeratinizing spinous cell carcinoma strain El.Cu. shown in FIGURES 30 and 31 grows very slowly in tissue culture, and can be maintained only with great difficulty. This strain has been maintained for over two and a half years. One wonders what the basic differences may be between this and the rapidly growing HeLa strain. There are, of course, many avenues of approach to this problem at the living cell level.

Heterologous transplantation of only one of these malignant cell strains (HeLa) in the guinea pig's eye has been demonstrated so far. Doctor Helene Toolan, of the Sloan-Kettering Institute, has demonstrated its ability to grow in cortisone-treated rats. A tissue culture recovery from our guinea pig passage of the HeLa strain has been isolated also. Its cytology and growth characteristics appear to be unaltered after a four-month sojourn in the heterologous guinea pig soil and with an estrogen pellet in the guinea pig (FIGURES 32, 33, and 28). Phase, cinephase, and electron microscope studies have revealed the presence of numerous microfibrils (FIGURE 29) on the surface of these cells, which correspond to the microfibrils (intercellular bridges) found in ultra-thin sections of carcinoma of the cervix. These and other cytological aspects are being studied.

The marked growth potentialities of the HeLa strain would appear to be related to the polyploid character of the cells (FIGURE 32) and to their aggressive feeding properties; both factors apparently contribute to their autonomous behavior. The irregular polyploidy is not greatly different from that found in many other cell strains that we have studied, to some of which reference has been made on previous occasions.[6, 27] Some investigators have claimed[28] that greatly enlarged hyperchromatic nuclei have only one ultimate fate, *i.e.*, autolysis. Our earlier work already referred to, as well as the current work on the HeLa strain (FIGURES 32 and 33), is not in agreement with such a conclusion, since very excellent motion pictures demonstrate the ability of even the most bizarre polyploid cells to divide and produce unequal-sized daughter cells, some of which are mixtures of aneuploids, diploids, or polyploids. The statements of Painter no longer hold on the question of the growth vigor of abnormal hyperchromatic cells.[28]

Summary

A few important highlights in the application of well-established tissue culture methods and new methods of advanced physical optics in wide use today as applied to some practical problems in virology and cellular biology are discussed. The isolation, management, use, and stability and instability (transformation) of strains of various normal and malignant cells are described. These valuable living biologic materials permit a variety of studies to be carried out which promise to advance our understanding of the biology of many diseases and to further our knowledge of growth processes at the cellular level.

Acknowledgment

We are deeply indebted to Peter Shapras* for his help in the production of the excellent phase photographs and for his help in the ultracentrifugation.

The examples of growth of strain HeLa cells were made from cultures main-

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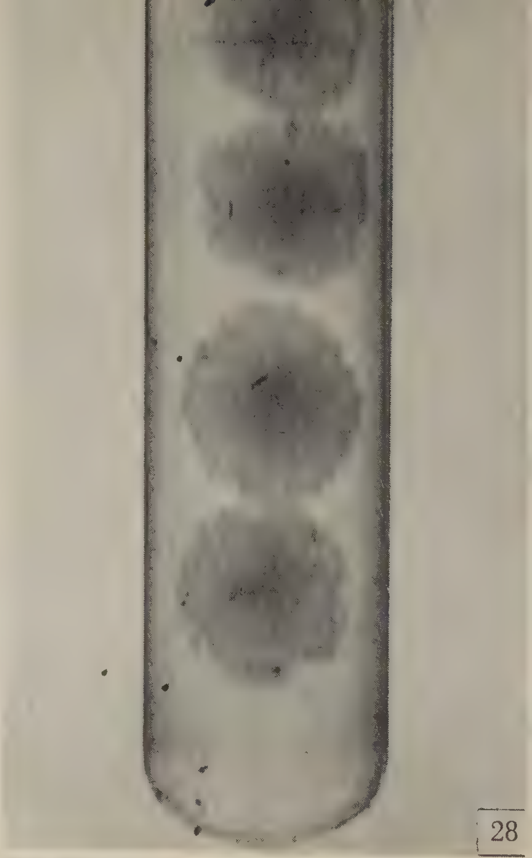
* Formerly Peter Sapranauskas.



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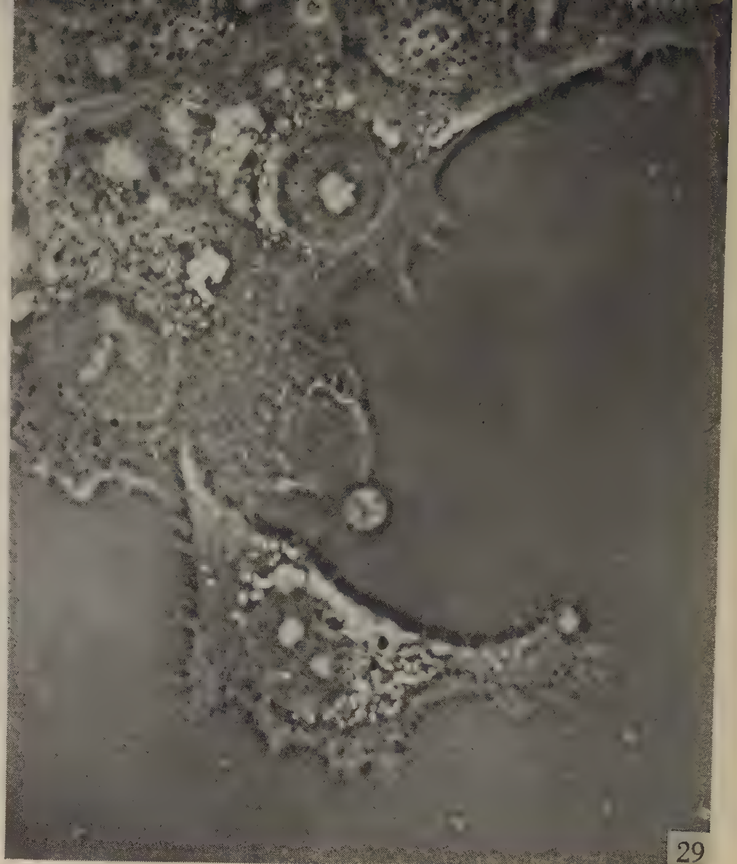

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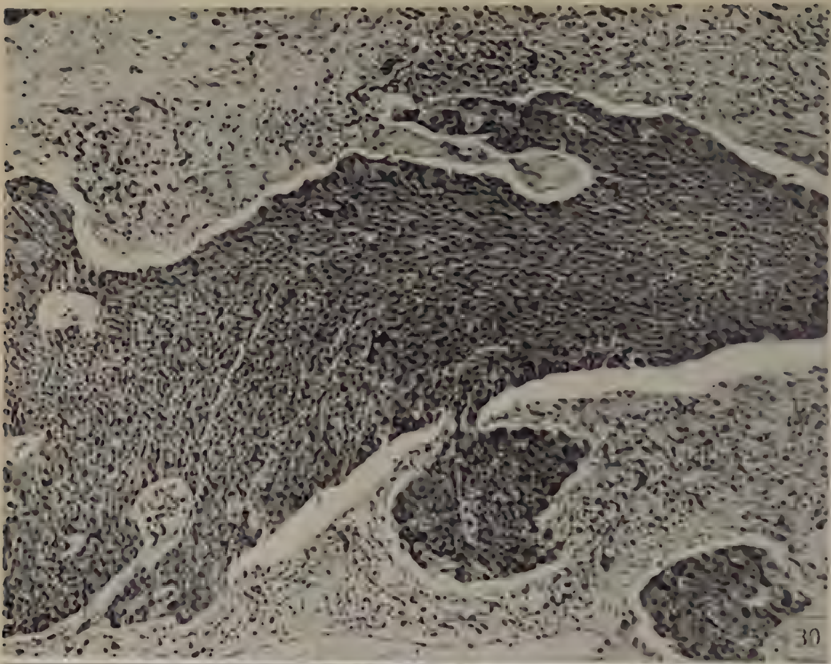
FIGURE 28 shows four large colonies of the HeLa strain of epidermoid carcinoma of the cervix 110 days after its recovery from a guinea pig's eye which was destroyed by its growth. Giemsa-stained roller-tube culture. Magnification ×2.

FIGURE 29 shows the character of the cells at the edge of the epithelial membrane of HeLa strain cells. The detached cell shows numerous fine microfibrils which can be interpreted as comparable to the structures producing intercellular bridges in vivo. Bright phase contrast. Magnification ×500.



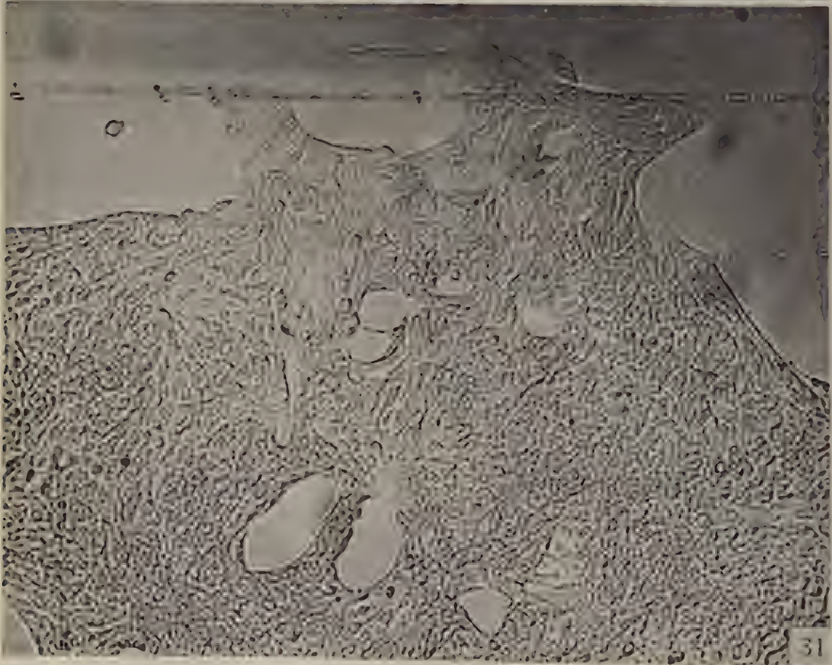
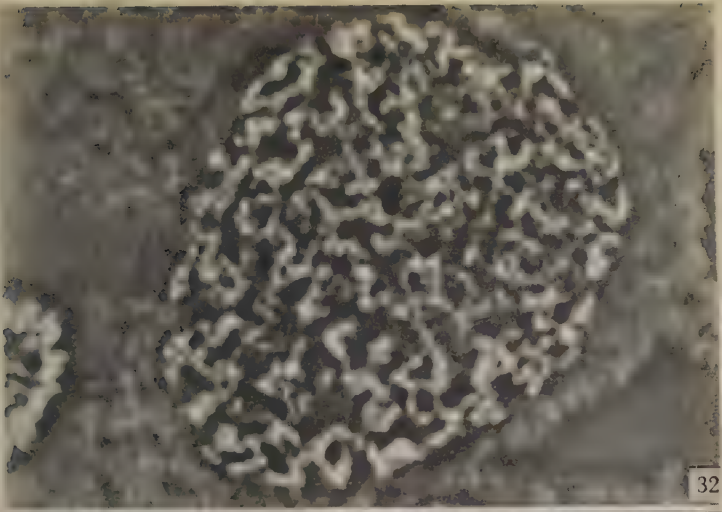


Figure 30 shows H and E stained section of spinous cell carcinoma from which strain ElCu was established.

Figure 31 shows a portion of a living colony of the ElCu carcinoma strain photographed after 313 days in continuous cultures. Magnification ×130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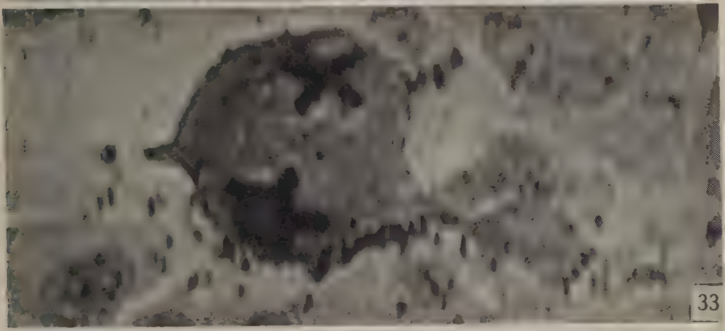
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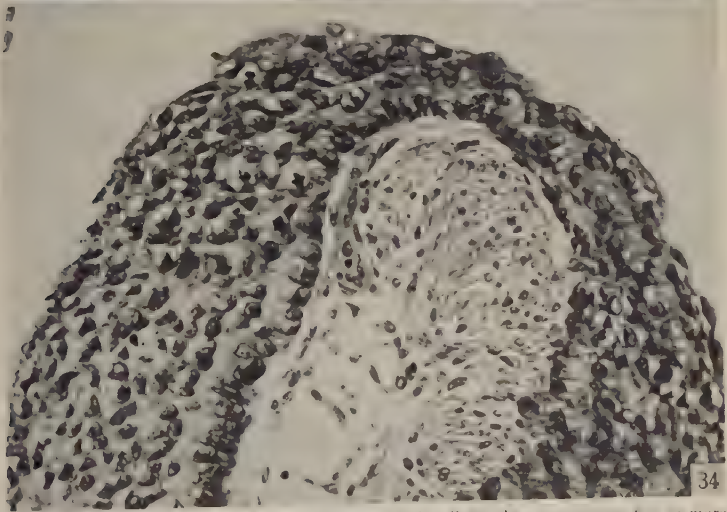
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FIGURE 32 shows a large polyploid prophase nucleus. Such cells produce as many as five or more unequal sized daughter cells. Aceto-orcein stain of 3-day slide culture. Cultured 447 days. Magnification ×1,800.

FIGURE 33 shows two tetraploid figures in one large HeLa cell from the guinea pig passaged strain 110 days in continuous culture since recovery from guinea pig's eye. Giemsa stained roller tube culture. Magnification ×550.

FIGURE 34 shows masses of HeLa cells surrounding a portion of the remaining iris of a guinea pig treated with estrogen. Note the palisading of the cells adjacent to the guinea pig stroma. Lesions in the retina showed marked invasion. H and E stained section about four months after implantation. Many dividing cells present. Magnification ×140.

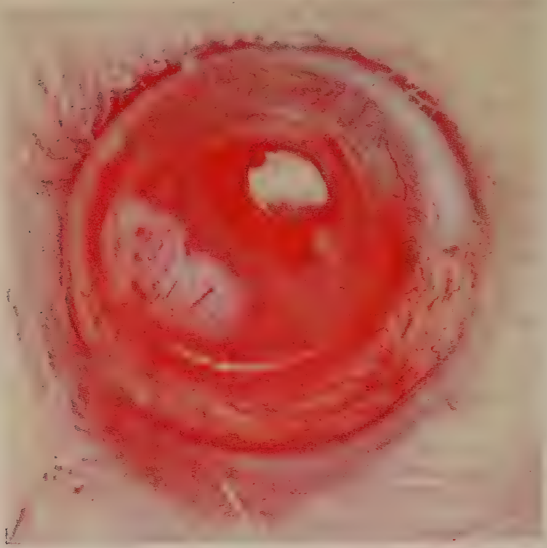

FIGURE 35. Colored photograph of large HeLa tumor mass well supplied with capillaries. About one month later, rapid growth and extension threatened to destroy the eye and involved the iris, lens, and retina.

tained by Doctor Maria Svotelis and originally explanted by Mrs. Mary T. Kubicek. Without the devoted efforts of these colleagues the results described in this report would not have been attained. We are grateful for their assistance.

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CERTAIN FACTORS LIMITING THE SIZE OF THE TISSUE CULTURE AND THE DEVELOPMENT OF MASSIVE CULTURES

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For the continued maintenance of tissue cells in culture outside the body, it is necessary that they be provided with nutrient materials suitable for their metabolism. From the observations of many workers, it has generally been accepted that, for satisfactory survival and proliferation of at least many if not all tissue cells in culture, the cells must also be provided with a solid substrate to which the cells may adhere and along the surfaces of which they may migrate.[1] Various types of substrates had been tried. The most widely used substrate, however, has been a thin layer of plasma or fibrin clot within which the cell clump was embedded. The form in which this plasma substrate culture was routinely used in our laboratory is shown diagrammatically in FIGURE 1.

A strip-shaped explant of tissue about ½ × 3 × 15 mm. in size was planted in a modified Carrel D-3.5 flask. This explant was embedded in a thin layer of solid plasma clot made up of plasma diluted with a mixture of horse serum, chick embryo extract, and balanced saline. After the clotting of this plasma or substrate, 1 ml. of the same dilute horse serum-chick embryo extract was added as a supernatant fluid layer. This supernatant was changed three times weekly. With a rapidly growing tissue, at 28 days the cell sheet had usually covered the floor of the flask and was transplanted by removing the sheet from the flask, cutting it into strips, and implanting each strip in a new flask. By this method of culture it was often possible to maintain cell strains in culture over a period of years.

This plasma clot substrate, however, had many disadvantages which interfered with the use of tissue cultures in accurate experimental work. The clot frequently clouded up, making optical study of the cells impossible. In all too numerous instances, the clot liquefied and destroyed the culture. The culture embedded in the clot could not be quantitatively separated from the clot for chemical analysis or for weighing. The chemically undefined nature of the clot limited exact work in cell nutrition and in chemistry. The cultures actually obtained with such a substrate were severely limited in size. For instance, with this type preparation, even with a rapidly proliferating cell strain, a culture which consisted of a thin cell sheet 33 mm. in diameter, probably containing less than 10 mgm. wet weight of cells, was to be considered most luxuriant.

As a result of the many handicaps and difficulties encountered with plasma substrate cultures, an attempt was made in our tissue culture section, at the National Cancer Institute to find some more satisfactory substrate. As the culmination of a long series of experiments, Evans and co-workers were able to show that excellent proliferation of numerous cell types cultured in a horse serum-chick embryo extract nutrient fluid could be obtained by growing the culture under and in contact with a sheet of finely perforated cellophane.[2, 3, 4, 5] Cultures planted in contact with this cellophane substrate proliferated luxuriantly and often reached an ultimate area and cell mass which far exceeded that attained in plasma substrate cultures. Instead of the small cultures obtained in the plasma clot, sheets of cells 60 square centimeters in area which contained over 100 mgm. wet weight of cells were frequently obtained (FIGURE 2).

Since this original successful application of the cellophane substrate, other studies from our tissue culture section have covered other phases of the behavior of cells grown on cellophane substrates. It was shown that, instead of transferring the culture by cutting out a fragment of the cell sheet and implanting it in a new culture flask with some cell strains, particularly with those which did not lay down a dense fibrillar network, the cells of the sheet could be shaken or scraped free from the substrate, suspended in a fluid nutrient medium, and planted as a cell suspension in the new culture.[5] Within a short time, the cells settled out on the cellophane, adhered to it, and proliferated as usual. It was later shown that, by using an even greater cell population density in the cell suspension inoculum, the cellophane could be omitted entirely from the culture flask (FIGURE 3).[6, 7] The cells settled out directly on the glass floor of

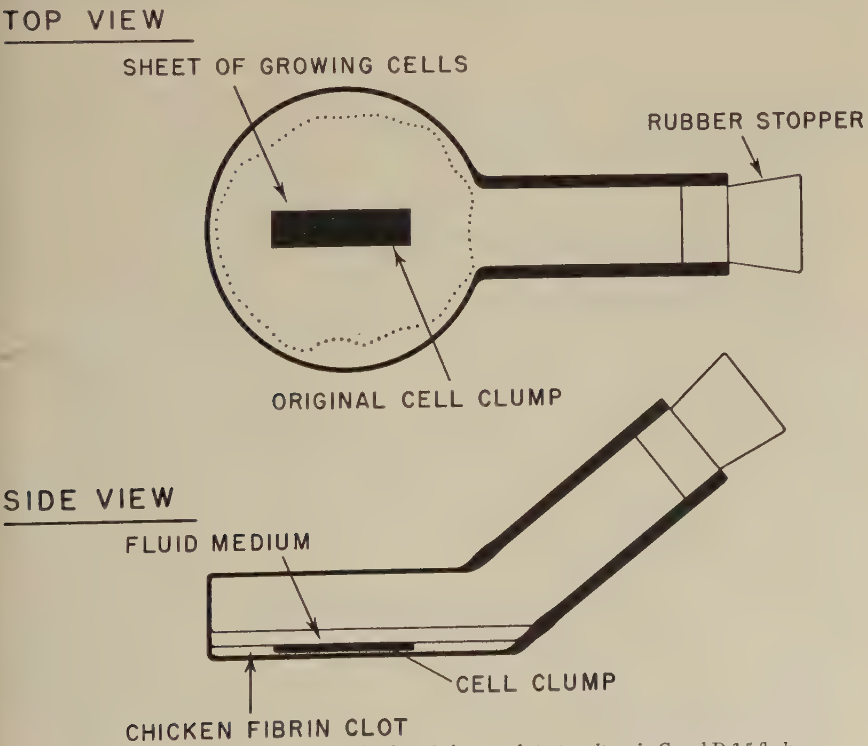


FIGURE 1. Diagrammatic top and side view of plasma substrate culture in Carrel D-3.5 flask.

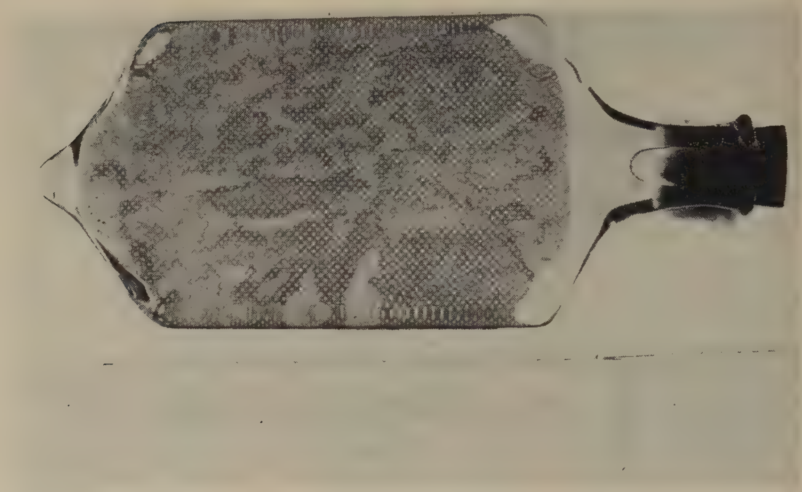


FIGURE 2. Photograph of fixed and stained culture of liver epithelium growing under a sheet of perforated cellophane in a T-60 flask. The stained material is the cells; the cellophane does not take the iron hematoxylin stain. The perforations of the cellophane are easily seen. The variation in density of stain in different areas is due to varying density of cells resulting from some wrinkling of the cellophane. The approximate area of cell growth is 60 sq. cm. The culture strain at this time was 335 days old and was fixed 12 days after implantation in this flask. This culture was implanted as a cell suspension.

the flask and proliferated there as luxuriantly as they had on the cellophane. From such cell suspension inoculum glass substrate cultures we have been able to obtain, in a number of instances, lots of from 1 to 10 gm. wet weight of cells for chemical study. With an established rapidly growing strain of cells we can usually count on from 75 to 100 mgm. wet weight of cells from each T-60 flask culture in from 12 to 14 days.

Two additional groups of studies are of particular concern today. Sanford and Likely carried out experiments in which single isolated cells from a strain of mouse subcutaneous fibroblasts, designated as strain L, were planted in capillary glass tubes.[8] When cultured in this way the capillary tubes apparently served to restrict diffusion of the medium around the cell, and gave each isolated cell a better chance to alter the culture medium directly adjacent, and so allowed the cell to build up a more optimal zone of altered or conditioned medium around itself. As a consequence, these workers were able to obtain proliferation of such isolated cells and to establish for the first time pure cell strains from them (FIGURE 4).

This method of isolation and of proliferation of single isolated mammalian tissue cells is apparently applicable to any cell type which can be maintained in rapid proliferation in a normal size culture.[9] To date nine pure cell strains, among which are included such diverse types as a mouse fibroblast strain (strain L, No. 929), a strain of mouse liver epithelium (No. 1469), a strain of human fascia fibroblast (No. 1814), and a pure strain from a culture which originally consisted almost or entirely of human skin epithelium (No. 1783) have been established and are now being maintained. As rapidly as these strains

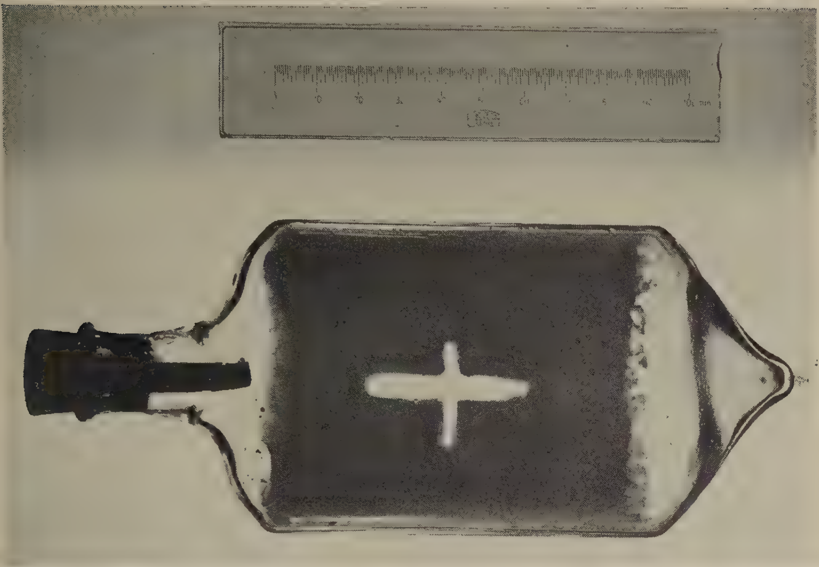


FIGURE 3. Unstained living culture of strain L No. 929 fibroblasts planted as a cell suspension growing directly on the glass surface of the flask. The cross in the center was scratched through the cell sheet to give a clearer concept of the density of the cell sheet.

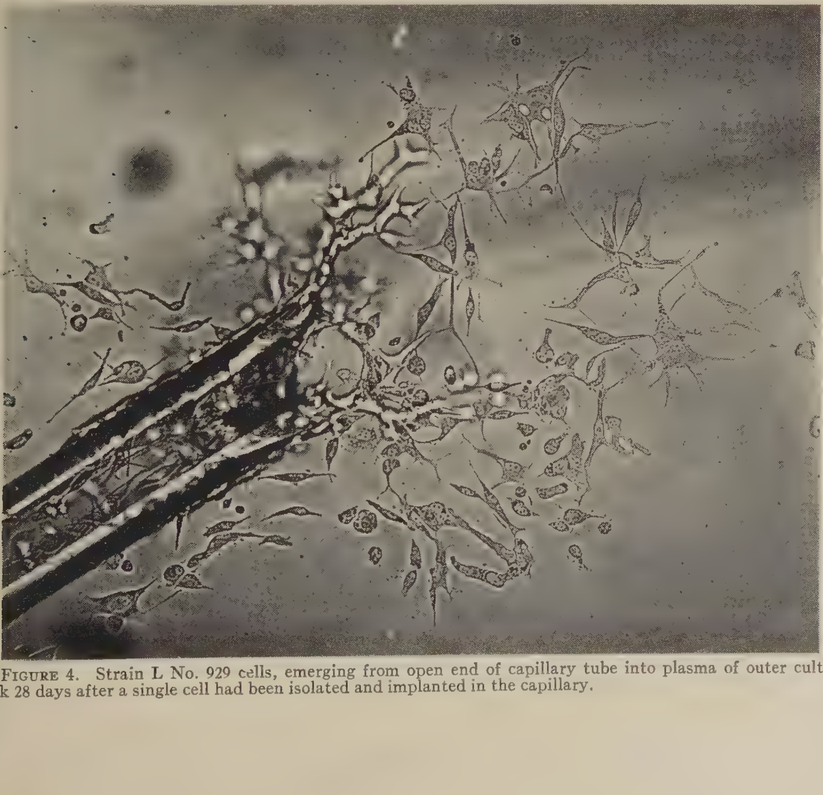


FIGURE 4. Strain L No. 929 cells, emerging from open end of capillary tube into plasma of outer culture flask 28 days after a single cell had been isolated and implanted in the capillary.

can be standardized and an adequate number of cultures obtained, they are being released to any recognized laboratory having facilities for maintaining them.

In addition to obtaining pure cell strains, the results of this study did indicate that even the best of our culture media, such as our usual chick embryo extract-horse serum nutrient fluid, was actually very poorly suited for the growth of tissue cells *in vitro* until the medium directly around the cells had been adapted or "conditioned" by the metabolic activity of the living cells.

Later quantitative methods were worked out by Evans, Sanford, and their co-workers,[10, 11, 12] for handling quantitatively the cell suspension used for planting new cultures. By these methods, large numbers of accurately replicate cultures could be planted from a cell suspension handled from a special burette. Equipment and methods were also worked out by which population changes in cultures of a group could be followed by enumerating the cell nuclei of cultures taken from such replicate culture groups.

This quantitative method of study, for the first time, allowed us to establish quantitatively that it was necessary to add a certain minimal number of cells as an inoculum to a given volume of fluid medium in order for the culture to survive.[13] For instance, with 2 ml. of the usual nutrient culture fluid made from chick embryo extract, horse serum, and balanced saline, an initial inoculum of strain L cells in the form of a cell suspension equivalent to from 100,000 to 500,000 nuclei resulted in proliferation of all cultures planted. An inoculum equivalent to from 10,000 to 50,000 nuclei gave only 64 per cent of the cultures proliferating, while an inoculum of cells equivalent to only 600 nuclei gave no proliferation. Instead, all cells rapidly died. These data seemed to confirm those obtained from the single cell cultures in indicating that optimal culture conditions required a certain alteration or conditioning of the medium by the action of the cells.

While the use of plane glass substrates, such as the glass surface of the flask, had allowed a great increase in the amount of cells which could be grown in culture, the plane substrate placed a practical limitation on the amount of cells which could be grown in the individual culture. An attempt was therefore made to build up a three-dimensional matrix[14] on the surfaces of which the cells could attach themselves, and through the interstices of which nutrient fluid and carbon dioxide-air mixtures could be periodically recirculated. Both folded cellophane matrices and matrices which consisted of masses of one-eighth-inch-diameter Pyrex glass helices were tried (FIGURE 5). The usual dilute horse serum-chick embryo extract nutrient fluid was used, and the cultures were inoculated with suspensions of pure strain L cells and of strain 1469 pure mouse liver epithelium. In all of the cultures tried with these types of three dimensional substrates, many cells could be seen adherent to the matrix surfaces, and the cell population, as determined by nuclear enumeration, rose far above the original inoculum level. In one culture of strain L on glass helices, for example, the final number of nuclei enumerated reached 174,280,000, estimated as in excess of 700 mgm. wet weight of cells. In another culture, on folded cellophane, the number of nuclei enumerated was 286,310,000, estimated as in excess of 1145 mgm. wet weight of cells.

From this first quantitative study we had made on cultures with a circulat-

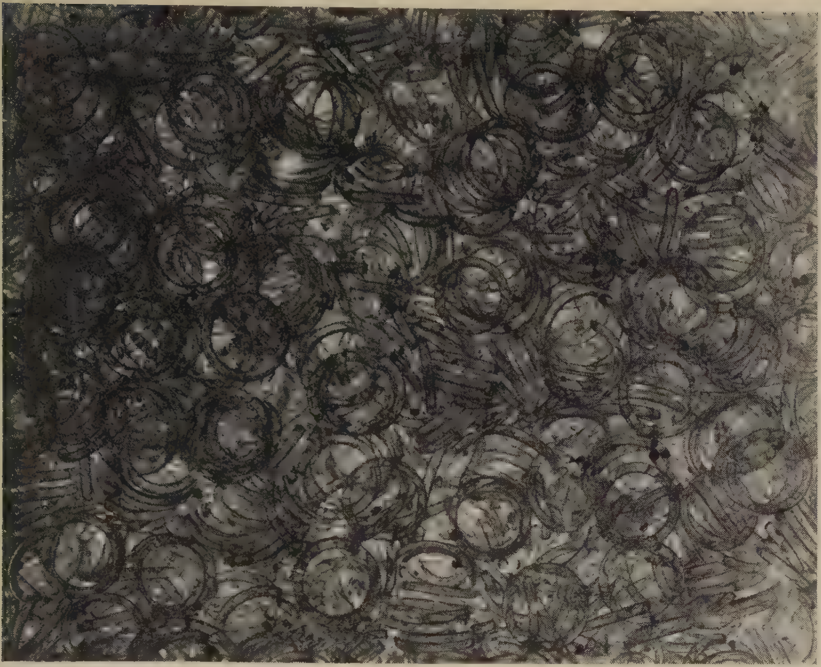
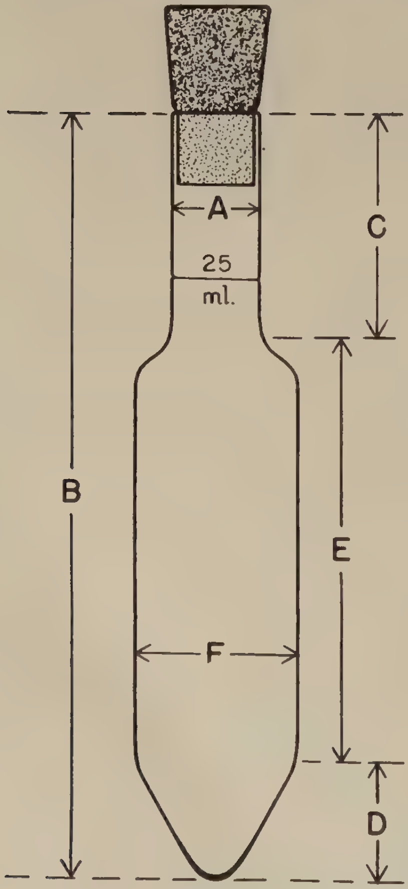


FIGURE 5. Packed Pyrex glass helices, as used for matrix substrate for growth of the cells. The central opening of each helix is about ⅛ in. diameter while the glass rod forming the helix is ½ mm. diameter.

ing medium, the question arose as to the influence of the rate of circulation of nutrient medium over the cells. Since the variables in the three-dimensional cultures were too complex for simple experimental analysis, the study of the influence of velocity of flow was undertaken by Schilling and Bryant on pure strain L suspension inoculum cultures planted in culture tubes of 22 mm. internal diameter and specially designed for nuclear enumeration studies (FIGURE 6). After planting, these tubes were kept rotating by incubating them in a multiple drum rotating or roller tube unit. This unit had eight drums, the rates of rotation of which were 9, 18, 37, 75, 150, 300, 600, and 1200 revolutions per hour respectively. Control cultures were also carried with cells in stationary tubes and in stationary T-15 flasks.

The significant point of this study,[15] so far as our present subject is concerned, is that the velocity of rotation of the tube, controlling the linear rate of flow of the nutrient over the inner surface of the tube, was a major factor in controlling the number of cell nuclei in the culture (FIGURE 7). By increasing the rate of rotation of the tube from the 6 to 9 revolutions per hour usually used with the roller tube drum, to 600 revolutions per hour, the level of nuclear populations was raised more than 80 per cent. Within the range of velocities studied, the optimal rate lay between 150 and 1200 revolutions per hour. Within this range, the population levels were so close together that no one culture rotation velocity can be considered as definitely superior over the whole culture period. There was no evidence that the cultures rotated at 1200 revolutions per hour were injured. In spite of these high velocities of nutrient fluid flow, it appeared that many or most of the cells were adhering to the surface of the glass tube.

In all of the preceding studies, a solid substrate to which the cells could adhere had been used. Earlier unpublished attempts in our laboratory to demonstrate cell proliferation in cultures in which the cells were freely suspended in a fluid culture medium and not adherent to some substrate had all failed. In spite of these failures, the frequent occurrence of dense cell suspensions in peritoneal ascites associated with carcinoma in the abdominal cavity suggested that cells might be made to live and proliferate in fluid suspension *in vitro*. The growth by Owens and Gey[16] of cells of a lymphoblastic mouse tumor in "tumbling tube" cultures *in vitro* has also suggested this possibility, although in this work there was a definite possibility that cell proliferation in fluid suspension *in vitro* might be a unique characteristic of cells of the lymphoblastic series. There were, therefore, no conclusive data to show that the fixed tissue cell.



DIMENSIONS
A = I.D. 10.3–10.8
A = O.D. 12.3–13.4
B = 115 mm.
C = 35 mm.
D = 18 mm.
E = 62 mm.
F = O.D. 25 mm.

FIGURE 6. Design of roller tube used in the study reported.

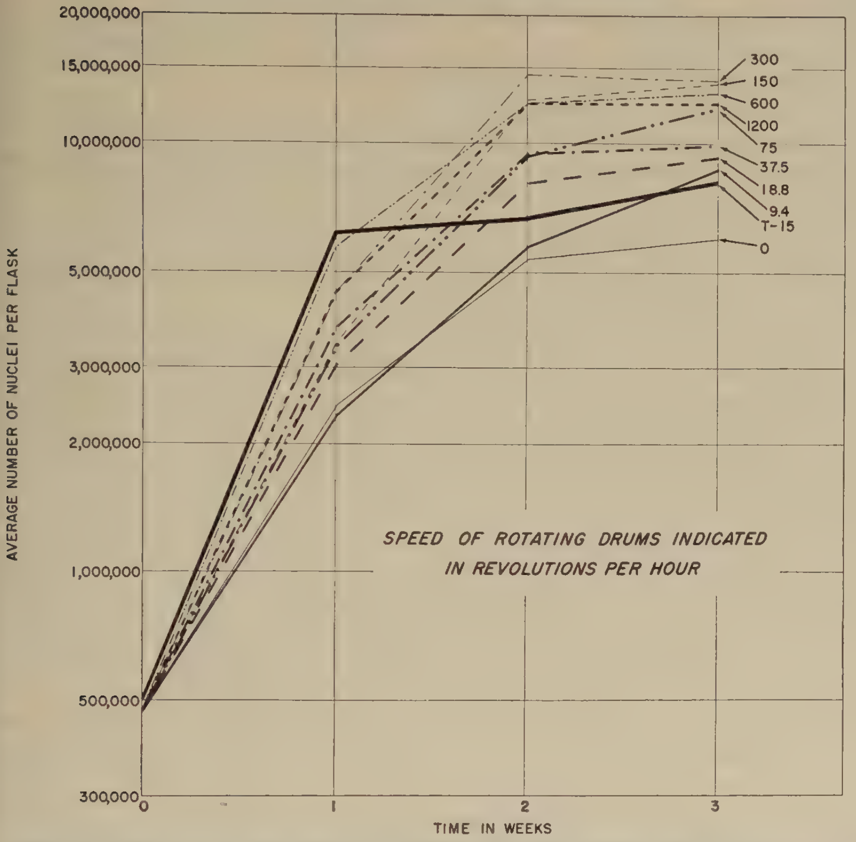


FIGURE 7. Influence of rate of circulation of the nutrient fluid on the population change of Strain L, No. 929 cells planted as a cell suspension in roller tube and in T-15 flask cultures. The line marked 0 is for stationary tube cultures; the T-15 line is for the T-15 flask cultures.

such as the fibroblast, could survive and maintain proliferation in culture *in vitro*, except when attached at some time to a surface substrate.

While the solid substrate culture could certainly be developed to large size, the advantage of being able to eliminate entirely the surface substrate as a complicating factor in the growth of large cultures and in experimental studies was very great. A new group of experiments to explore the possibility of obtaining rapidly proliferating cultures with the cells freely in suspension in the nutrient fluid was therefore started.

In planning a restudy of this problem of growing tissue cells in fluid suspension cultures, several factors were given special attention.[17, 18] These were:

(1) It was obviously necessary to keep the cells in suspension in the nutrient fluid. To accomplish this result, roller tube velocities of up to 2400 revolutions per hour were tried. To assist in this procedure an extremely slight increase in the viscosity of the nutrient fluid by some such means as the addition of a low concentration of hyaluronic acid or methylcellulose could be effective. Since

hyaluronic acid compounds were not available in pure form, methylcellulose in low concentration was used. In the final experiments, a concentration of 0.1 per cent was used in all nutrient solutions.

(2) It was necessary to maintain adequate circulation of the culture fluid. With three-dimensional surface substrate cultures, earlier experiments had shown that an adequate rate of circulation of the nutrient culture fluid over the cells was of major significance for continued rapid cell proliferation. With a deep layer of a fluid suspension of cells, for rapid cell proliferation, a rapid circulation of the fluid was certainly necessary in order to insure adequate oxygenation of the cells. Concurrently, rate and manner of circulation must be such as not to injure the cells mechanically.

(3) It was necessary to start the culture with an adequately dense cell population in the form of a cell suspension. Previously reported work on the growth of isolated single cells had indicated that even the best fluid culture media now available must be altered or "conditioned" by the cells before rapid cell proliferation occurred. Owing to the necessary rapid circulation of the fluid in the suspension culture, with constant washing of fluid over all surfaces of the cells, it was probable that the actual necessary stage of conditioning of the nutrient fluid was extraordinarily critical and might require for attainment a very high initial cell population density.

Since our earlier unsuccessful study of fluid suspension cultures had been made with cells of pure strain L No. 929 fibroblasts, cells of this same strain were used. The usual fluid medium of dilute horse serum and chick embryo extract was used.

This attempt to proliferate the strain L No. 929 cells in fluid suspension has been successful (FIGURF 8). After an initial period apparently required by the cells to adapt or condition the fluid culture medium, the cells established and maintained a state of rapid proliferation in the fluid suspension cultures during the course of the experiments. In cultures of intermediate and higher rotation velocities, that is at 600 to 2400 revolutions per hour, there was no evidence of cell attachment on the surface of the glass tube, as determined by microscopic examination. With intermediate velocities of rotation, that is, at 150 to 600 revolutions per hour, the cells aggregated in small granular or spherical masses from 0.1 to 0.5 mm. in diameter, suspended in the nutrient fluid. With higher velocities of rotation, the cells remained suspended as independent single cells and as cell aggregates of up to about 10 cells. The free-floating cells were rounded up, spherical in shape.

At this point in our study, we know that at least one type of fixed tissue cell could be grown in fluid suspension cultures. We were impressed with how closely the factors involved paralleled those which had been encountered in the growth of fungi in the antibiotics industry. If our concepts were right, it appeared that equipment and methods designed for use in the antibiotics industry could be used for the growth of animal tissue cells. In order to test this concept on a small scale, a New Brunswick type shaker* was obtained (FIGURE 9) and was mounted in an incubator. In different experiments, this

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* New Brunswick Instrument Co., New Brunswick, N. J.

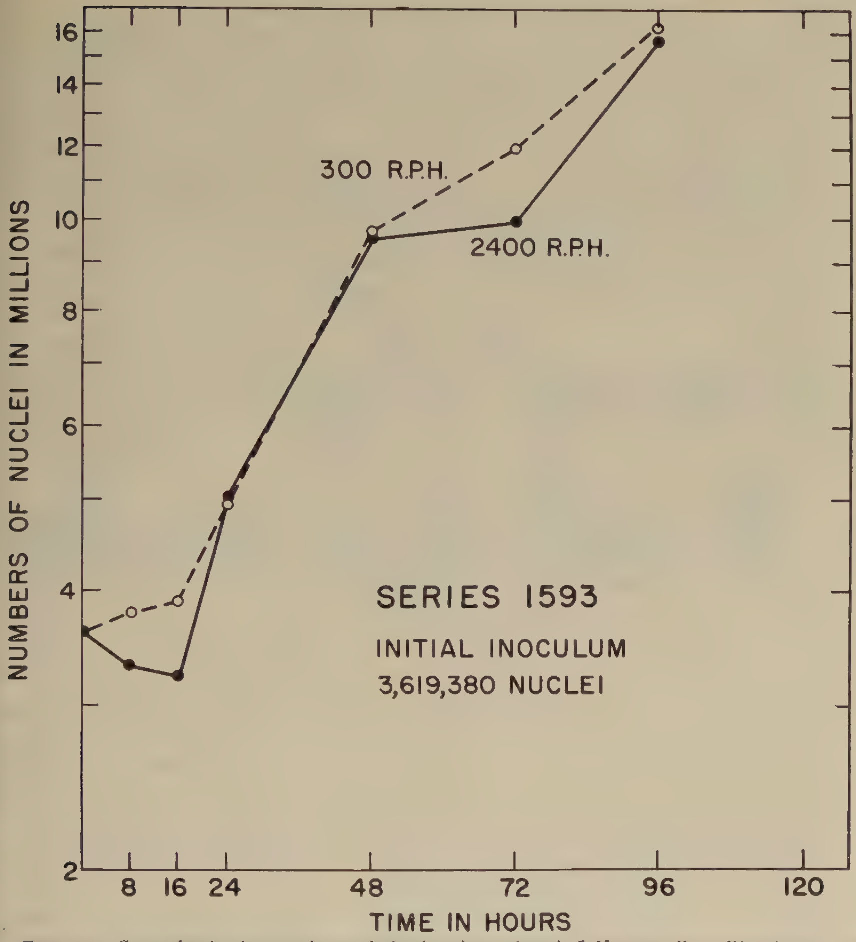

FIGURE 8. Curve showing increase in population in culture of strain L No. 929 cells proliferating as a cel suspension free-floating in the nutrient fluid.

instrument was adjusted to shake at between 4800 and 10,000 revolutions per hour. On this shaker, to date, using the fluid culture medium described for the preceding study, we undertook to grow strain L (No. 929) fibroblasts and cells of pure strain (No. 1469) of mouse liver epithelium. Our attempts have been successful.

With strain L (No. 929) fibroblasts, for instance, cultures were each started with cells equivalent to 4,200,000 nuclei suspended in 10 ml. of fluid culture medium, each culture contained in a 125 ml. Erlenmeyer flask. Thrice weekly, the cell suspension was lightly centrifuged, most of the supernatant removed and replaced with fresh fluid nutrient. As the volume of fluid increased, the culture was removed to a larger flask until, at the end of 25 days, the 300 ml. of cell suspension was contained in a one-liter flat-bottom boiling flask. At

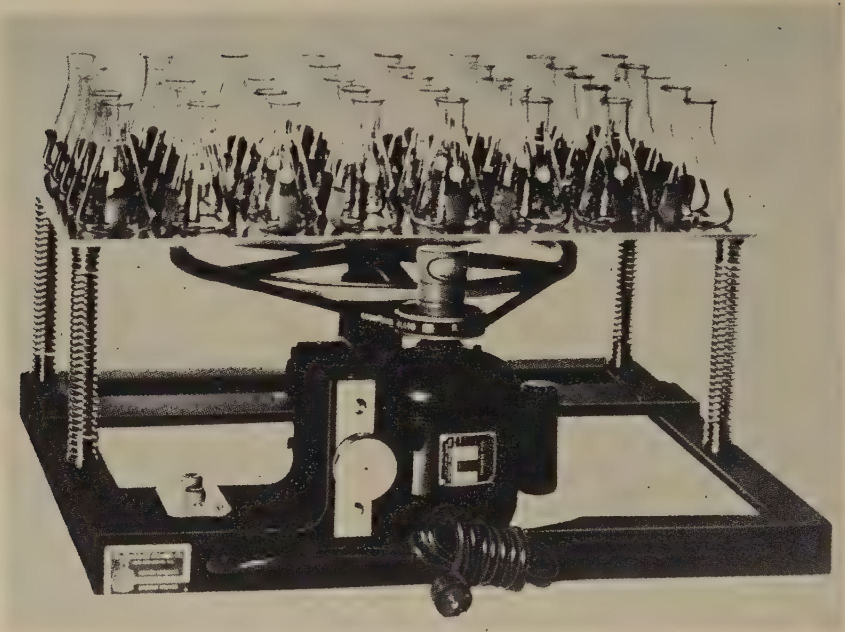

FIGURE 9. Brunswick type shaker used in the study reported.

this time, enumeration of an aliquot showed the culture contained approximately 370 million nuclei.

At the end of 25 days, this culture was subdivided into five cultures which were each started in 80 ml. of nutrient. This subdivision therefore started each daughter culture with cells equivalent to 72,000,000 nuclei. Some of these cultures were closed out for study, but those which were run on were each proliferated to cells equivalent to 400,000,000 nuclei, or an estimated wet weight of more than 1.5 gm. wet weight of cells each, in seven days.

Rapid proliferation of cells under these experimental conditions has also been confirmed for cultures of the No. 1469 mouse liver epithelium, but since data on this strain are less quantitatively detailed, they will not be presented here.

Certainly the experimental conditions used in the preliminary experiments so far carried out on cultures of both of the cell strains could be much improved. In particular, it appears essential to have mechanisms for continuously compensating rapid pH drift owing to acid production, and to insure an adequate supply of oxygen in the culture vessel. From the data obtained, it is our impression that these changes in the culture system will greatly accelerate cell growth. Even so, from the nature of the factors involved, it is reasonable to expect that other types of fixed tissue cells can also be grown in fluid suspension cultures. As adequate types and amounts of nutrient media become available, there appears to be no reason why such growth of animal tissue cells in fluid suspension cultures should not be carried out, if desired, on a scale comparable to that used in producing fungi in the antibiotics industry.

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STUDIES OF CERTAIN DEFINED MEDIA FOR TESTING PROLIFERATION IN REPLICATE TISSUE CULTURES*

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In the tissue-culture laboratory of the National Cancer Institute, the earliest effort toward solving the problem of replacing a naturally occurring medium was directed toward replacement of the plasma substrate, since it severely limited exact work in cell nutrition. Cells imbedded in a plasma clot cannot be separated for chemical analyses, for weighing, or for counting, and their proliferation is limited. With the introduction of the use of perforated cellophane,[1] some of these problems have been resolved. The cellophane, when used with fresh tissue, helps to hold the explant and also the cells of the young culture in position so that they spread freely over the glass and cellophane.

Cultures carried for many generations under cellophane grow so profusely that they routinely cover 60 sq. cm. in T-60 flasks.[2] For our nutrition studies,[3] several such cultures of the single cell Strain L fibrosarcoma cells are scraped or shaken from the surface to form a suspension which is lightly centrifuged. The centrifugate, after being washed with saline and one of the fluids common to the media to be tested, is passed through a double mesh platinum alloy sieve to break up any cell clumps. This washed suspension is received in a specially designed funnel where it is stirred with a glass stirrer at 450 rpm. to distribute the cells uniformly throughout the fluid. While being stirred continuously, the cells are delivered in aliquots to individual culture flasks.

A quantitative procedure has been developed so that the numbers of nuclei may be estimated readily with a high degree of accuracy in both the original inoculum suspension and later in cultures subjected to experimental treatment. Either an aliquot of the cells in the inoculum suspension or those in the culture under assay conditions is treated first with 0.1 M citric acid solution in order to strip the cytoplasm from the nuclei and then with crystal violet to stain the nuclei for easy identification. These nuclei are then counted in a 0.1 mm. deep haemacytometer chamber.

As judged by subsequent growth of the cultures,[3] these cells stirred in suspension at 450 rpm for 20 minutes are not injured. At periodic fluid changes, such cultures can also be safely centrifuged at 350 rpm. and when the special fluid removal apparatus[3] is used, insignificant numbers of cells are lost from the cultures. Proliferation curves of the averages of the numbers of cell nuclei per flask, determined by the quantitative enumeration procedure, are plotted on a semilogarithmic grid against time of exposure to the assay material. Slides of the graphs to be shown subsequently were plotted in this way. These combined procedures of preparing replicate cultures and enumeration of nuclei thus provide a technique to follow accurately changes in cell population as indicated by changes in nuclear numbers in cultures exposed to assay conditions.

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Two major approaches have been used in our laboratory to pursue the nutrition studies. The first of these, which is the physical fractionation of the two natural components of tissue culture medium, horse serum, and embryo extract, has given much useful and practical information. The second approach has been the gradual building up of a defined medium composed of materials commercially available and known or suspected to have some nutrient value for cells *in vitro*. This approach has resulted in a medium satisfactory for maintaining cells over a short interval.

In the fractionation studies of the serum and embryo extract presently under consideration, both the large molecular residues that fail to pass through the membrane of a Simms ultrafiltration apparatus and protein free ultrafiltrates were tested on cells to determine whether any of these fractions might be substituted for the unfractionated horse serum or the unfractionated embryo extract.

The horse serum residue[5] used with chick embryo extract was found to be much more effective in increasing numbers of nuclei than the horse serum ultrafiltrate used with the embryo extract. The extent of proliferation, however, was lower than in the control cultures grown on unfractionated horse serum and unfractionated chick embryo extract.

Chick embryo extract ultrafiltrate and chick embryo extract residue were also tested. It was found that the ultrafiltrate could replace the whole extract.[5] In summarizing the results of these experiments, it may be seen that Strain L cells will proliferate in the absence of the complex protein and nucleoprotein residue fraction of embryo extract. This finding is contrary to the generally accepted view that the major growth-promoting properties of embryo extract lie in the fractions of high molecular weight.

The effects of the four fractions of embryo extract and horse serum were observed in various combinations on replicate cultures.[5] Greatest proliferation with fractions was obtained when chick embryo extract ultrafiltrate and horse serum residue were combined. This proliferation, however, was somewhat lower than that in the control cultures.

The replicate culture procedure has also been used to learn the effect on cells of two of the more promising chemically defined mixtures[6] recorded in the literature. We hoped to learn if both or either of these mixtures could be used as a basal nutritional medium adequate to maintain washed suspensions of Strain L fibrosarcoma cells in replicate cultures close to or at a given inoculum level. In these experiments, replicate cultures of 500,000 cells per flask were exposed to a mixture for 6 and 11 days. Six combinations were tested and compared: (1) balanced saline; (2) saline and chick embryo extract; (3) White's chemically defined medium; (4) saline, chick embryo extract, and horse serum; (5) Morgan, Morton, and Parker's chemically defined mixture 199[7] prepared at the National Cancer Institute; and (6) mixture 199 prepared at the University of Toronto. The mixtures were not basal media according to the definition as given in this paper.

Media composed of parts of one of these mixtures,[7] together with chick embryo extract ultrafiltrate and horse serum residue fractions, produced a significant result[8] on replicate cultures. Supplementation of these two fractions with 40 per cent by volume of the amino acid constituents of mixture 199 provided a culture fluid for growth of the replicate cultures superior to the control medium of horse serum and chick embryo extract.

Further work is being done to replace both or either of these two undefined media fractions of embryo ultrafiltrate and horse serum residue by substituting a chemically defined medium. Simultaneously, work is being done to fractionate further the two effective fractions and to make extensive chromatographic analyses of the fractions.

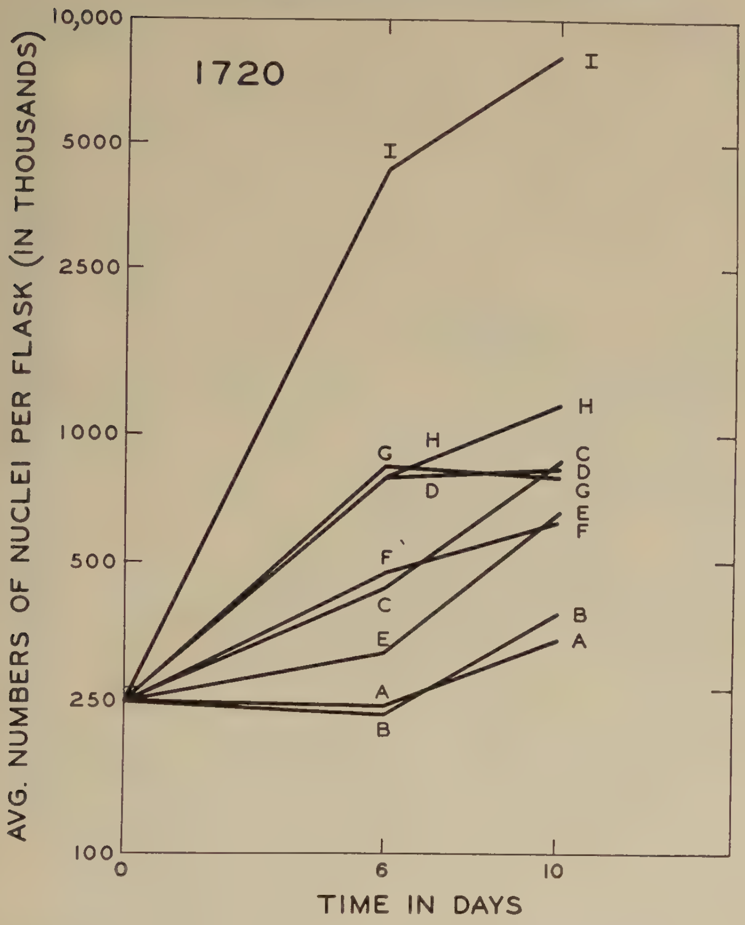
At the same time that the assay of the various fractions of whole medium was being made on cells, Doctor Westfall[9, 10] was making chromatographic analyses of the two ultrafiltrates for their amino acid content. About 26 ninhydrin-positive substances appeared in the serum ultrafiltrate and were considered to be amino acids or amines. In addition, two peptides were present. The ultrafiltrate of the chick embryo extract also contained the same amino acids and amines, but apparently in somewhat different proportions. In addition, it contained at least three peptides, two of which appear to be different from those of the horse serum, and perhaps two additional unidentified ninhydrin-positive materials.

Doctor Westfall's analyses have served as the basis for our amino acid mixture NCTC 101 which we are using as a component in the media of our more recent experiments.[11] In these experiments we have investigated the effect on proliferation of methocel 4,000 centipoises, methocel 15 centipoises, peptides, ovalbumin, globulin, dextran, coenzyme concentrate, and a combination of glutathione and ascorbic acid.

In experiment 1720, several combinations of materials (FIGURE 1) were compared with negative and positive controls. In half of the eight media, methocel was included at a constant concentration. Embryo extract ultrafiltrate was in all the media, as well as the mixture of amino acids, in tenfold concentration, vitamins, *etc.*, designated as NCTC 102.[11]

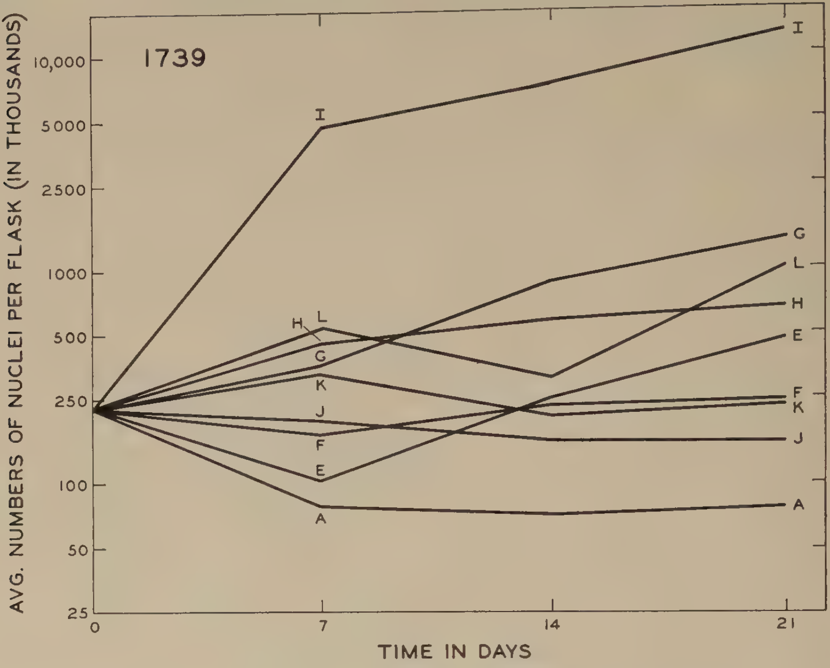
Selection of the particular peptides was governed by the need, first, to use those commercially available and, secondly, to include those containing as many different amino acids as possible within the limits of solubility requirements. The nitrogen content of this mixture was nearly the same as that of the horse serum which the peptides were replacing. FIGURE 1 shows that these peptides cannot substitute for ovalbumin. The peptides at a 20 per cent level were less effective than either 20 per cent ovalbumin or a combination of 10 per cent peptides with 10 per cent ovalbumin. Ovalbumin in the medium increases proliferation, whether at a 20 per cent level or 10 per cent level. The difference between the 20 per cent level and the 10 per cent level is not significant. The presence of 4000 centipoises methocel in a medium increases proliferation and the effect is present on both the sixth and tenth days.

In the experiment 1739 (FIGURE 2), the effect of the peptide mixture was reinvestigated; the effect of bovine gamma globulin on proliferation was considered; and additional experiments were run on the effect of methocel. Methocel again had a significant effect, although the result is based on a small com-



	A	B	C	D	E	F	G	H	I
Embryo extract ultrafiltrate	20	20	20	20	20	20	20	20	20
Amino acids, vitamins, etc. (NCTC 102)	40	40	40	40	40	40	40	40	40
Peptides (NCTC 103)	–	20	–	10	–	20	–	10	–
Ovalbumin, 6.4 %	–	–	20	10	–	–	20	10	–
Methocel, 4000 cps, 0.5 %	–	–	–	–	20	20	20	20	–
Horse Serum	–	–	–	–	–	–	–	–	20
Saline	40	20	20	20	20	–	–	–	20

FIGURE 1. Proliferation curves of tissue cultures of experiment 1720 over a 10-day period.

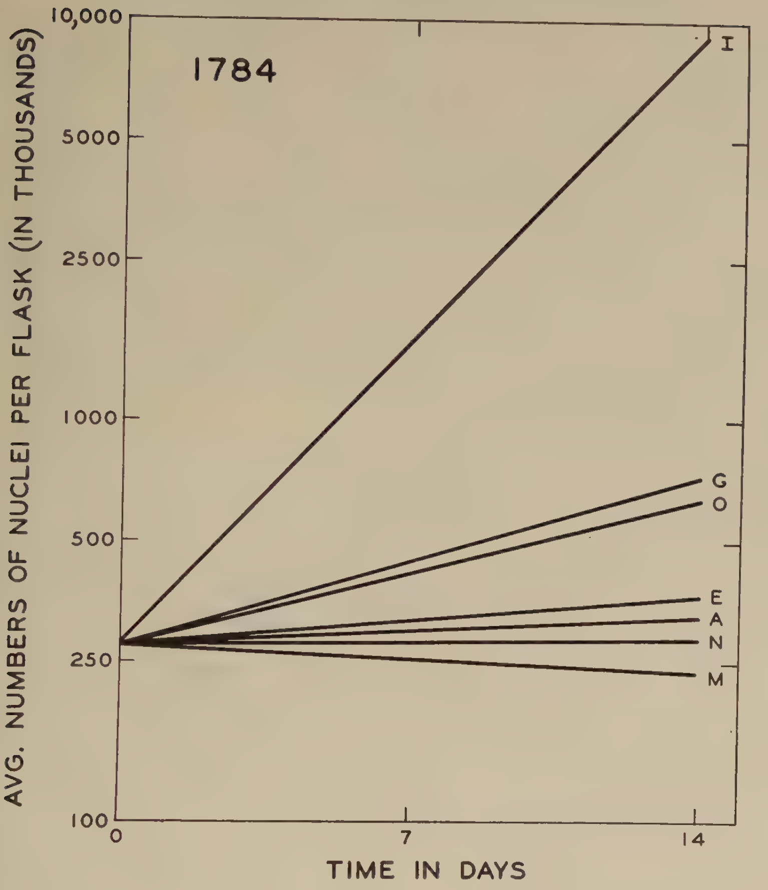


	A	E	F	G	H	I	J	K	L
Embryo extract ultrafiltrate	20	20	20	20	20	20	–	–	20
Amino acids, vitamins, etc. (NCTC 102)	40	40	40	40	40	40	40	40	40
Peptides (NCTC 103)	–	–	20	–	10	–	10	–	10
Ovalbumin, 6.3 %	–	–	–	20	10	–	10	20	–
Methocel, 4000 cps, 0.5 %	–	20	20	20	20	–	20	20	20
Globulin, 6.5 %	–	–	–	–	–	–	–	–	10
Horse Serum	–	–	–	–	–	20	–	–	–
Saline	40	20	–	–	–	20	20	20	–

FIGURE 2. Proliferation curves of tissue cultures of experiment 1739 over a 21-day period.

parison. The peptides again proved to be ineffective as a substitute for either the ovalbumin or the globulin. However, the bovine gamma globulin could substitute for the ovalbumin. There was no significant difference in the amount of final proliferation of the cultures in either case. In this experiment, we prepared one mixture, series K, that was free of embryo extract ultrafiltrate and contained twice-crystallized ovalbumin as its only major source of protein nitrogen. This medium maintained the cultures over the three-week period. We consider it to be a basal medium as we defined such a medium earlier in this paper.

Experiment 1784 (FIGURE 3) continued investigation of substitutes by including some new variables and some of the old for reaffirmation. No further



	A	E	G	I	M	N	O
Embryo extract ultrafiltrate	20	20	20	20	20	20	20
Amino acids, vitamins, etc. (NCTC 102)	40	40	40	40	40	40	40
Ovalbumin	–	–	20	–	20	20	20
Methocel, 4000 cps, 0.5 %	–	20	20	–	–	–	–
I. Methocel, 15 cps, 3.75 %	–	–	–	–	20	–	–
II. Methocel, 15 cps, 3.75 %	–	–	–	–	–	20	–
Dextran, 6 %	–	–	–	–	–	–	20
Horse Serum	–	–	–	20	–	–	–
Saline	40	20	–	20	–	–	–

FIGURE 3. Proliferation curves of tissue cultures of experiment 1784 over a 14-day period.

examination of the effect of peptides or gamma globulin was made. Since dextran has been used as a plasma substitute, we tested 6 per cent dextran, mol. wt. 25,000 to 200,000, as a methocel substitute. Dextran is about the equivalent of the 4000 centipoises of methocel in its effect on increasing proliferation. Within 14 days, 15 centipoises of methocel prepared in two duplicate lots did not support proliferation above the inoculum level. The cultures exposed to these duplicate preparations were not significantly different. Final population was three times as high on 4000 centipoises of methocel as on 15 centipoises, even though the two solutions were prepared so that the final viscosities of the media to which the cells were exposed were closely equivalent. The mixture containing 4000 centipoises of methocel again showed a positive effect on the proliferation of the cells over that seen when it was omitted. As has been demonstrated with the preceding experiments, the inclusion of ovalbumin was decidedly effective.

In experiment 1791 (FIGURE 4), only two new combinations, series P and Q, were introduced. It was necessary to carry over some previously investigated series to have adequate controls, one of which was series K, initially introduced in experiment 1739, and containing ovalbumin but no embryo extract ultrafiltrate. In the present experiment, it behaved in the same manner as series P and Q, to the first of which coenzyme concentrate was added; to the second, glutathione and ascorbic acid were added. By the seventh day, series K, P, and Q were about equal although below series C and G. The best of these series, P, was very little different from series G. By the fourteenth day, a striking difference was seen between the series with embryo extract ultrafiltrate and those without it. The result of a comparison between cultures with and without coenzyme concentrate, *i.e.*, series P as compared with series K, suggests that the concentrate is not of great positive value in these experiments, though different concentrations probably should be tested.

Again the addition of methocel to the mixture in the presence of embryo extract ultrafiltrate showed a favorable action on proliferation by the 14th day. While the difference between the series with methocel and the series without methocel is not statistically significant in this particular experiment, it is a positive effect. This is consistent with the previous experiments in which a positive (and statistically significant) effect was found.

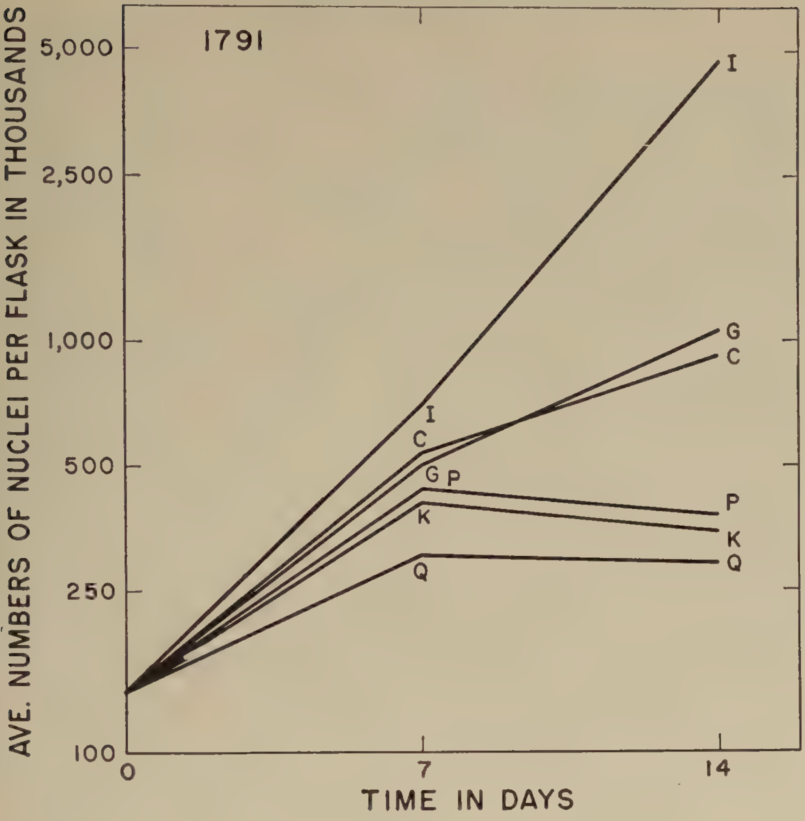
We have tried here to demonstrate the approaches which we are using in an endeavor to obtain a defined or reproducible medium capable of maintaining rapid and extensive proliferation of cells *in vitro*.

We have been able, so far, to quantify our handling of cultures.

We have fractionated the natural media, horse serum and embryo extract.

We have found that methocel and dextran have a positive effect on proliferation and are equal in behavior; but dextran is no better than methocel and it is probably not as reproducible commercially from one lot to another.

Ovalbumin is effective in a medium, and so is gamma globulin, though the latter is no better than the former and it is not as readily available and reproducible commercially so that twice-crystallized ovalbumin is a material of choice over gamma globulin.



	C	G	I	K	P	Q
Embryo extract ultrafiltrate	20	20	20	–	–	–
Amino acids, vitamins, etc. (NCTC 102)	40	40	40	40	40	40
Ovalbumin	20	20	–	20	20	20
Methocel, 4000 cps, 0.5 %	–	20	–	20	20	20
Coenzyme concentrate, 1 mg. / 100 ml.	–	–	–	–	20	–
Glutathione and Ascorbic Acid	–	–	–	–	–	20
Horse Serum	–	–	20	–	–	–
Saline	20	–	20	20	–	–

FIGURE 4. Proliferation curves of tissue cultures of experiment 1791 over a 14-day period.

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Discussion of the Paper

Doctor Raymond C. Parker (*Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada*): I should like to congratulate Doctors Earle, Bryant, Evans, Sanford, and their colleagues, for their very great contribution in devising the replicate culture assay procedures, and I should like to acknowledge our own debt of gratitude to Doctor Earle's group for its kindness, a year ago last summer, in helping us set up the techniques in our Toronto laboratory. Before that time, we had to assay our synthetic media with fresh tissues in roller tubes and had to depend on periods of survival in order to judge the effect of different substances added to the media; but as the media improved, these assays took longer and longer to complete and finally had to be abandoned as routine procedures in screening new compounds. I should like to suggest, however, that Doctor Earle and his colleagues are unduly pessimistic about the future of synthetic media, by which I mean chemically defined media unsupplemented with naturally-occurring ingredients. My colleagues and I now have, in our Toronto laboratory, a basal synthetic medium, No. 703, in which we are able to obtain, in seven days, five- and sixfold increases in the population of replicate cultures prepared from washed suspensions of Earle's L strain mouse cells. At the moment, we also have cultures of L strain cells that have lived and multiplied for over 140 days in synthetic medium 703, with two complete renewals of the medium each week. These cultures are very similar in appearance to long-term cultures of the same cells cultivated in horse serum and balanced saline.

I should like to summarize the steps that were taken to bring about these improvements in the synthetic media. Because we were worried about the high oxidation-reduction potential of our earlier medium, No. 199, we attempted to bring it nearer the physiological range by increasing, very considerably, the levels of the three reducing substances, cysteine, glutathione, and ascorbic acid. When these substances were tested individually, at the higher levels, the two containing the SH groups increased the rate of cell multiplication and improved the appearance of the cells, whereas ascorbic acid had no apparent effect. When it was found that cysteine and glutathione could be used interchangeably, cysteine was chosen because it was less expensive and was incorporated at 2600 times the level used in solution 199. Then, ascorbic acid was added at 1000 times its original level in the hope that it would help maintain the SH compounds in the reduced state. Finally, glutathione was increased to 200 times the old level because it is now known to be a coenzyme. This medium was designated No. 612. Next, in order to study the effect of certain nucleic acid constituents, a new basal medium (No. 635) was devised by omitting from solution 612 all the purines (adenine, guanine, xanthine, and hypoxanthine), the pyrimidines (thymine and uracil), as well as ATP, adenylic acid, ribose, and desoxyribose. This medium gave a much better growth response than solution 612, but although three- to fourfold increases in the cell population of L strain cultures could be obtained in seven days, the cultures rarely lived longer than 40 days. We did use it to excellent advantage, however, in testing individual purines and pyrimidines, individual ribose and desoxyribose nucleosides and nucleotides, intact DNA and RNA and their enzymic hydrolysates, and even homologous DNA prepared from the L strain cells themselves. Then, several months ago, we undertook a study of certain coenzymes and made preliminary tests with Armour's crude coenzyme concentrate from pork liver. When this material was added to solution 635, at levels of 1 or 2 mgm. per cent, it yielded five and sixfold increases in the cell population in seven days. The crude concentrate was known to contain CoA, DPN, and TPN; but when these three coenzymes, in a purified state, were added individually to solution 635, they had no effect. When they were added in combination, however, to give solution 703, they caused approximately the same growth response as had been obtained with the crude concentrate. Still more important, from our standpoint, is the fact that L strain cells in solution 703 are alive and healthy, and are undergoing continuous cell multiplication after 140 days. The details of this work have already been prepared for publication by G. M. Healy, D. C. Fisher, and R. C. Parker, in the Canadian Journal of Biochemistry and Physiology.

CHEMICALLY DEFINED SYNTHETIC NUTRIENTS FOR TISSUE CULTURES FOR PHARMACOLOGICAL STUDIES

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Doctor Waymouth and I have been asked to discuss the development and use of synthetic nutrients for tissue cultures in the study of pharmacological products. Since our paper is the first one to deal explicitly with pharmacological problems, we envisage our role as including the definition of terms, the outlining of concepts, and the setting forth of limits and boundaries as we see them. Having done this much, we shall review briefly what we ourselves have been able to accomplish to date within these limits, in the hope that such a summary may prove useful to our colleagues.

Pharmacology (or materia medica, as it is still sometimes called) comprises the study of substances of biological or artificial origin applied to disturbed or pathological states in the organism. Until quite recently, this study was the province of the botanist and herbalist rather than of the chemist. Today, there remain in the pharmacopoeia very few natural products whose essential active components have not yet been chemically defined. The subject is now almost coextensive with chemotherapeutics. Modern therapy with synthetic drugs and antimetabolites is based more and more on the rational development of specific agents, and less and less on empiricism.

While all pharmacological products are designed to affect pathological or disturbed states, they do so in a variety of ways. In fact, a given substance may be a drug in certain circumstances, but not in others. Foods and vitamins are not ordinarily drugs. But sugar is a drug in the treatment of acute insulin shock; ascorbic acid in the treatment of scorbutic eczema; thiamin in the treatment of beriberi. The classification of a substance as a pharmacological agent depends on its usefulness in relieving a pathological state.

Drugs may be classified broadly into at least four groups:

First, there are substances which directly affect the subject, either generally or locally. Nicotinic acid is a directly acting general drug, while digitalis and pyribenzamine are drugs of localized effectiveness.

Second, there are substances which have a preferential effect on infective or parasitic agents. The antibiotics and antihelminthics are such substances. Penicillin acts directly on the invading organism. These drugs affect pathological states of "external" origin.

Third, there are products which are intended to exert a preferential effect upon malignant cells without affecting the tumor-bearing host. (Unfortunately, the metabolism of tumor cells differs very little from that of the host tissues and such differences as do exist are not yet well understood. We believe that tissue culture techniques are well adapted to improving our understanding of these differences. Until this understanding is greatly improved, however, a rational tumor therapy is hardly practicable.)

Fourth, there is a group which acts indirectly, through the subject or host, by a series of steps, so that the drug itself plays no part in the final end effect. Vaccines are examples of this class.

Tissue culture can be useful in studying drugs acting in some of these ways, but quite useless in other respects. We need to be quite clear about the limitations of the technique. We must beware of leading our medical colleagues to expect too much of tissue culture assays. Pharmacological actions in which the body itself contributes to the effect of the drug are mostly unsuited for study in isolated cell systems in culture. We may study the direct effects of cortisone on collagen formation *in vitro*, but the effect of cortisone in alleviating arthritis through stimulation of the endocrine glands which, in turn, influence the pathology of the articulations is totally unsuited to *in vitro* study unless perhaps by breaking it down into a series of separate component stages. Tissue culture is useful principally in studying drug effects which are local, at the cellular level. Other collateral techniques are available for studying drug reactions at other levels, in isolated organs, or in the intact animal.

Since tissue culture assays must generally be limited to cellular responses, we must choose a series of such responses by which modes of functioning and degrees of effectiveness are to be assessed. At least three possible criteria are evident:

(1) We can investigate specific metabolic changes produced by the agent tested, in relation to dose and time. This is a well-established approach in the study of bacterial systems; *e.g.*, of the reactions of bacteria to antimetabolites. It is relatively untried in pharmacological assays with metazoan cells, although an interesting example, the direct action of vitamin A upon cell function, will be described by Doctor Fell later in this monograph.

(2) Ideally, we should like to be able to discern the specific effects of pharmacological products on individual metabolic processes. We are as yet seldom able to do so. Usually, we can measure only over-all effects upon cell metabolism, in terms of toxicity. We can ask "Will a particular product kill a culture of cells or selectively attack certain cells in a mixed population? How soon will it do so and at what minimum concentration? Can the cells recover from sublethal dose-times and, if so, is recovery complete and how promptly will it supervene?" These are clear-cut criteria and relatively simple to apply. But even when we have answered these questions we must remember that there is no simple rule for referring toxicity, as determined *in vitro*, to a corresponding toxic dosage *in vivo*. The latter depends, among other things, upon the distribution of the drug in the organism, its solubility in body fluids, and the rates at which it is detoxified and excreted.

(3) We may investigate the effects of drugs on the particular series of processes which become visible in mitotic proliferation. These effects may include stimulation or inhibition of mitosis, blocking mitosis at a particular stage, and production of chromosomal abnormalities.

Let us consider how we can set up tissue culture systems in which these criteria of pharmacological action can be effectively applied.

It is 23 years since Carrel stated that " . . . cells are in physiological continuity with their environment. Cells and environment form a whole. A cell depends as strictly upon its medium as the nucleus upon the cytoplasm."

This dependence is very important to remember, because, whereas *in vivo* the environment of any cell is the resultant of an immensely complex system of biological mechanisms which serve to maintain physiological "normality," *in vitro* the environment which we provide is an artificial one, subject to experimental rather than biological regulation. In tissue cultures, the culture medium and the external incubation conditions, with the cultured cells, constitute a closed system which governs completely the nature and the degree of the response which the cell presents to the introduction of any variable. A pharmacological agent may be looked upon as such a variable. Since no artificial environment is "normal," we must arbitrarily set up physiologically acceptable standards upon which to impose this variable. The more precisely the environment can be defined, in physical and chemical terms and still remain capable of supporting a function which we can agree to regard as normal, the more confidently can we translate our results *in vitro* into terms of successful application *in vivo*.

How far do present-day culture practices satisfy these requirements of precise definition of the environment coupled with the physiological normality of the cells? The type of environment most often chosen is designed to promote a high rate of "growth," which may mean rapid cell migration, high mitotic rate, short period for doubling cell number, increase in weight, or high metabolic turnover. This concept represents a very limited view of physiological normality. It represents fairly accurately the state of a regenerating tissue, but it does not at all represent the state of healthy, functioning body cells. Should we not seriously consider other standards of normality? Is not prolonged function at a low level of mitotic activity a physiological characteristic of equal importance? Are not heart muscle and neurone, which do not normally proliferate, equally suitable material for pharmacological study? Should we not consider the desirability of setting up environments suitable for their study as well as for proliferating tissues?

Whether in practice we are to study a pharmacological effect *in vitro* over a short period or a long one, we should aim at conditions which, if we wished, could permit the experiments to be extended over fairly long periods. We believe that prolonged survival, prolonged persistance of mitosis, even at low levels, prolonged capacity to resume growth upon application of a suitable growth stimulus or transfer to a growth-promoting environment, constitute a group of basic criteria of physiological function of real importance. Our success will depend on the degree to which prolonged function can be combined with precise control. We should also aim at a third goal, the *simplest* possible constellation of conditions which will satisfy the other two requirements.

Most of the current tissue culture media are intended to support a high level of proliferative "growth." To accomplish this result, they are provided with serum, plasma, and tissue extract. These three products are subject to wide biological variation from one batch to another, and are reproducible only to a very gross approximation. Moreover, the very complexity and all-inclusiveness of materials such as serum and tissue extract obscure the finer nutritional requirements of specific cells. As the nutritional requirements of cell types are defined with greater precision, different standard "physiological" media will be required for cells of different origin. It is our thesis that more flexible and more precise standards of normality can be established on the basis of media of chemically defined composition than upon media containing a multitude of undefined and variable components.

An example can be given of the difficulties which may result from failure to control the biological unknowns in an experimental system. In some of the early work on the cobalamins, assays for haemopoietic capacity were made in tissue cultures of human bone marrow. These cultures were initially prepared in diluted human serum. The difficulty was to find a medium which did not itself promote megaloblastic maturation. Untreated pernicious anaemia patients are, of course, no longer to be found. Normal human serum contains maturing factors. Here was a clear case for the use of a defined medium, free of maturing factors.

Biological media which promote active proliferation, in spite of their failure to satisfy our demands for control and constancy of composition, nevertheless have certain advantages for the routine maintenance of large colonies of cells for use in assaying tests. We have ourselves used such media with embryonic and malignant cells. The most generally used nutrient of this kind is that variously defined by Carrel, Gey, Earle, Hanks, and others, consisting of 40 per cent of some balanced salt-dextrose solution, 40 per cent of serum of some sort, and 20 per cent of embryo extract. Any of a number of salt-dextrose solutions may be used—Tyrode, Pannett and Compton, Gey, Earle, Hanks, White. The serum may be chick, horse, or human. The embryo extract may be chick or beef. All will permit a high level of proliferative growth for a fairly wide spectrum of normal and malignant animal cells. We have used such media to build up and maintain routine cell colonies; however, we have sought to extend the boundaries of control by reducing the concentration of unidentified substances. One of us (PRW) is at present using routinely a nutrient consisting of 80 per cent salt-dextrose solution (White), 10 per cent horse serum, and 10 per cent of embryo extract. This nutrient permits cell multiplication at a level almost equal to that obtained in the 40:40:20 formula and has served to maintain Earle's strain L cells for many months. Parker, in maintaining another strain of cells, has reduced the embryo extract to 1 per cent, but has retained a high level (39 per cent) of serum.

Our aim is to establish simple conditions, precisely controlled, permitting prolonged function. Very simple conditions indeed can be used as a starting point. When cells are suspended in a balanced salt-dextrose solution without supplements of any kind, some cells may survive for six to eight days. The addition of eight other chemical compounds (ascorbic acid, cysteine, glutathione, and five amino acids) about trebles the survival time; *i.e.*, extends it to more than 20 days. A more complete amino acid mixture (15 amino acids) raises the survival to 30 days. The addition of another two compounds (hypoxanthine and glutamine) to 20 in all, besides the salts and glucose, extends this period to more than 50 days. Such media are still relatively incomplete, but this sort of experiment demonstrates that survival is in no sense dependent on the use of complex biological nutrients of ill-defined composition. Still more adequate and complete media have been devised. White's W_{II} nutrient, with 38 components, permits a still longer survival, to more than 80 days. A greater number of components does not, however, necessarily improve survival. Parker's formula 199, which has come into rather general use, contains about 60 ingredients yet does not permit any longer survival of similar cells.

Moreover, we no longer need to resort to complicated mixtures containing serum and embryo extract in order to study proliferating cells. Two years ago, one of us (CW) tested a relatively simple nutrient which consisted of a salt-dextrose solution supplemented with amino acids and peptides. This medium promoted a high level of mitotic activity for several days followed by rapid degeneration. A later formula containing 31 components has supported mitoses in chick heart cells for 27 days. Parker has graciously furnished us with the formula of his defined nutrient No. 703 before formal publication. This nutrient contains 58 ingredients and has supported the proliferation of Earle's strain L cells for 112 days, with active mitoses. We now have a stock of Earle's strain L cells of about 100 flasks, every culture of which is derived from cells which have passed through at least one three-week period on a completely defined nutrient; some of them have had 90 days or more, in two stages, in synthetic nutrients.

We believe that some of these defined nutrients which we are using, relatively simple though they are, are still more complex than necessary. These nutrients contain 16 or more amino acids and numerous vitamins whose functions have not been studied in detail. Some of these components are probably unnecessary, or are needed only to supplement synthetic processes of the cell which are inadequate for optimal function. We shall be surprised if the total number cannot be reduced to 20 or less. This total is still quite a formidable number of variables, controllable though they are, upon which to impose an assay substance, but it is diminutive in comparison with the number of components in an uncontrolled biological nutrient of the classical kind. We believe that it is worth emphasizing that the greatest single improvements we have made in the past two years have come from reexamination of such supposedly well established factors as the phosphate-bicarbonate buffer system and the sugar content of the nutrient.

While these simple, defined nutrients are adequate to support prolonged survival, relatively normal metabolism, and low-level mitotic activity for several types of cells, what advantages do they possess for pharmacological studies over the biological media now in general use? The advantages are certainly many. It has long been recognized that chick embryo extract, when used in the study of virology of chick tissues, or even in general biological studies, presents a distinct hazard of introducing unrecognized chick viruses into the background. The problems of obtaining serums of sufficiently reproducible properties, bearing in mind the wide differences, for example, between serums from young and old animals, are obvious. The problems of autologous versus homologous versus heterologous serums are well known. The problems of constancy of supply, availability of donors of uniform age, nutritional state, *etc.*, are known. Statistical averaging by use of mixed supplies is no real answer. The Tissue Culture Commission set up its testing laboratory as an attempt at standardization of some of these variables in biological materials. The problems involved are obvious, long recognized, and more or less successfully dealt with in a variety of individual, empirical ways.

There are other problems which are less commonly faced. In studying antispasmodic drugs, one needs to know and control precisely not only the calcium level of the background solution, but also any possible traces of biological agents such as parathyroid hormones, epinephrine, *etc.* In studying the effects of carcinostatic or carcinogenic agents, the possible presence of natural carcinogens, procarcinogens, or cocarcinogens in the form of body sterols or other substances, should be excluded as far as possible. In studying vitamin and hormone effects, one should define the background concentrations of these substances. Perhaps, in these cases, the levels present in the embryo extract and serum are too low to be significant. Often we do not know or neglect to take the known values into consideration. The question becomes particularly acute where the substance in question is important at extremely low concentrations. Cobalt as a factor in the vitamin B_{12} pattern is one of these. How much cobalt is there in the serum of a horse raised on Maryland bluegrass? Is such a serum significantly different from that of a Nebraska horse raised on prairie grasses? What is the copper content of a Maine-bred chick embryo in February as compared with a beef embryo obtained from the Chicago stock yards in August? These may seem exaggerated questions, but they are real and significant ones.

Chemically defined nutrients now available are simple in composition and capable of supporting prolonged survival, relatively normal metabolism and function, and low-level mitotic activity of a variety of cells. We believe that these nutrients possess real superiority for pharmacological work over biological media, in terms of greater precision in defining the conditions which serve as background for experimental studies on the effects of drugs on living cells.

THE CERTIFICATION OF COMMERCIAL CULTURE MEDIA

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It is generally recognized that there is a great deal of time-consuming routine involved in preparations required for the *in vitro* culture of tissue cells. Quantities of glassware must be carefully cleaned and sterilized, and media of several types have to be elaborately processed. Since a similar routine is practiced in all cell culture laboratories, the total effort invested in these straightforward technical aspects of tissue culture becomes enormous. In an attempt to relieve this situation, and so give the investigator more time for cell studies, the Tissue Culture Commission, a few years ago, undertook to encourage commercial houses to begin the preparation and sale of culture media. As its part in the project, the Commission agreed to advise on preparation procedures and to examine and certify the products. The report that follows is designed to give a brief account of this latter aspect of the project.

To begin with, a small testing laboratory was set up at the Sloan-Kettering Institute in space generously provided by its director, Doctor C. P. Rhoads. Financial support was obtained from the American Cancer Society via the Committee on Growth of the National Research Council. Under these auspices, the laboratory started functioning in October 1948, and continued until July 1950. The actual testing and analyses of media were done by Miss A. J. Hollander and Mrs. Enid M. Barry, and what success the project achieved must largely be credited to their skillful and painstaking efforts. Difco Laboratories, Inc., in Detroit, Mich., and Microbiological Associates, Inc., then of Coral Gables, Fla., cooperated on the commercial side.

It was hoped at the outset that the services of the testing laboratory would not be required beyond two years. This period was considered adequate for the commercial laboratories to establish a reliable routine of procedures and their own system of production controls. Progress was slower than anticipated, however, and, even at the end of this period, the commercial product was not by any means perfect. Nevertheless, a beginning had been made and, in the succeeding four years there has been a steady increase in the use of such materials.

The procedures for media preparation which were recommended to the producers were those in most general use in culture laboratories at the time. Naturally, the producers were interested in rendering such media as stable as possible so that they could be stored over long periods of time or shipped to consumers without appreciable deterioration. In this connection, the work of Hetherington and Craig[6, 7] had demonstrated that certain types of media such as plasma and embryo extract could be lyophilized and reconstituted for culture purposes without apparent loss of essential properties. This method of preservation was accordingly employed wherever possible. As will be explained below, the lyophilized material gave the consumer certain problems, especially with regard to plasma, and so it should be mentioned that subsequent experience (in tests made by Microbiological Associates, Inc.) has demonstrated that plasma and other materials which are altered by lyophilization may be stored and shipped to distant points while in the frozen state.

The testing routine, which is more properly the interest of this Conference, was carefully worked out with other members of the Tissue Culture Commission and, of course, it made use of the techniques and experience then available. Nowadays, different procedures would be adopted, though I am not sure that the results would be greatly different or more meaningful.

In the first place, it was early recognized that the testing program could aim to do little more than detect frank toxicity or *sub*standard properties in the commercial products. To attempt more would have required a larger laboratory than it was possible to operate. In this limited aim, then, each commercial sample was compared with an equivalent material freshly prepared in the testing laboratory. The commercially collected and lyophilized plasma was thus tested or rated against a freshly collected, nonlyophilized standard plasma, and similar standards were used for each item presented to us for examination. Except for lyophilization, the formulas and procedures used in making the standards were identical with, or as similar as possible to those used in the commercial laboratories.

The test object was the cultured cell. The different media components presented to the laboratory were examined in a variety of ways, as indicated below, but to determine their worth as media for tissue culture, reliance was placed chiefly on the behavior and growth of cells subjected to them. Originally, it had been planned to use a particular strain of cells and measure its growth response by chemical determinations of the increase in nucleic acid phosphorus. However, these plans proved to be impractical. Too much time and effort would have been required to keep the cell strain in sufficient supply and, after a trial run, it was easily calculated that, if the growth were measured by such chemical determinations, the laboratory could not in a reasonable period examine all the samples submitted to it. Rather than delay test reports and thus retard progress in the manufacturing end of things, it was decided to use fresh tissue explants for each test. These explants were derived from a uniform source (the thigh muscle of a 14-day-old chick embryo) and growth was measured in terms of colony diameter. Measurements were made after 96 hours in culture and, at the same time, any abnormal appearance in the cells was noted.

Admittedly, this procedure was crude, but when the culturing was done by a single operator with carefully measured and uniform quantities of media and, when the comparisons between controls and tests were made by the same observer, remarkably slight differences were detectable and significant. The worth of the procedure was evident in the excellent uniformity shown by the cell colonies grown under identical conditions.

It was necessary, in any case, to be content with such a test, and there seemed little reason to doubt that media thus examined and found equal to the laboratory control would be satisfactory for most types of tissue culture. This procedure left to the investigator of unusual problems in tissue culture the necessity of finding out for himself whether the commercial products would be useful for his purposes.

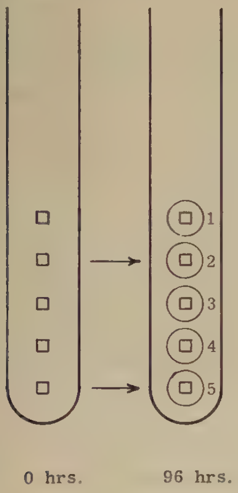
Summary of Testing Procedure

Test object: Explants (approx. 2 mm. x 2 mm.) of skeletal muscle from thigh of 14-day-old chick embryo.

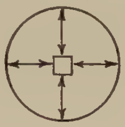
Test cultures: 3 roller tubes with 5 explants each for control media.

3 roller tubes with 5 explants each for test media.

Test period, method of measurement and tabulation of data:



Average radius of each colony measured with ocular micrometer.



Tube	Explant No.					Average per tube
	1	2	3	4	5	
1	150	145	150	140	150	147
2	145	140	150	150	145	146
3	135	145	140	145	145	142

Average for 15 test colonies = 145

Average for 15 control colonies = 143

$$\text{Growth index} = \frac{\text{test}}{\text{control}} = \frac{145}{143} = 1.0$$

Figure 1. This figure summarizes procedures used in making culture cell tests of commercial media. Explants of skeletal muscle derived from the thigh of 14-day-old embryos constituted the test object. These explants were cultured in roller tubes, five explants to the tube, and three tubes (or 15 explants) were used for each test. After four days incubation in contact with the test material, the colony diameters were measured, averaged, and compared with those of cell colonies grown in the presence of the standard or control material of the same type. The ratio of these averages, referred to as the growth index, was taken as a measure of the worth of the commercially prepared material. Whenever the ratio was below 0.85, the medium was considered sufficiently substandard to be rejected.

Figure 1 summarizes the testing procedure as applied to a single sample of, let us say, embryo extract. In every case, six culture tubes were prepared, each planted with five uniform pieces of tissue in a thin film of plasma. These were incubated for two hours at 37.5° C., to allow for complete coagulation and syneresis of the plasma clot. The extract to be examined was then introduced as a nutrient fluid into half the tubes, and the freshly prepared equivalent was placed in the other three tubes. The cultures were next incubated for 96 hours without change and then examined. Colony diameters were measured, tabulated, and averaged as indicated, and the average diameters of colonies in the test material were compared with those in the control. The ratio was taken as the "growth" index of the test material and became the value which determined whether or not the commercial product would be certified. Whenever a value of 0.85 or less was obtained, the material was considered unsatisfactory and was rejected.

Each medium component was examined in an appropriately different manner as indicated in TABLE 1. The object in each case was to achieve as intimate a contact as possible (*i.e.*, consistent with cell survival and growth) between the cell and the material being tested.

In the case of (a) *plasma* the explants were placed in a mixture of equal parts plasma and balanced salt solution (b.s.s.). This test was designed to detect any toxicity in the plasma. At the same time, cultures of the skeletal muscle explants were set up in Carrel flasks in a mixture of one part test plasma and two parts of a standard nutrient mixture 5:3:2 (five parts b.s.s., three parts cord serum, and two parts embryo extract), for the purpose of determining whether the plasma would show any unusual tendency to lyse under the influence of active cell growth and colony development.

Likewise for (b) *embryo extract*[*] the tests were devised for the purpose of detecting toxicity as well as growth-promoting properties. The former attempt was made by setting up tissue explants in a thin plasma substrate and then, after clotting, adding the test and control embryo extracts as supernatants. A 20-per cent extract (20 per cent of EE_{20})[†] was used. The growth-promoting properties of the commercial sample compared with the control extract were observed by comparing the above cultures relative to one another and to a culture without extract. A similar measure of its properties, when incorporated in a complete medium, was obtained by adding the standard 5:3:2 mixture, including the test extract, to explants in the thin plasma substrate.

The (c) *balanced salt solutions* were tested by placing them alone [except for thin plasma (12 per cent) coagulum] in contact with the tissue explants and developing cell colonies.

And so also (d), the *various sera* were examined, first, for manifestations of toxicity by using them alone as the fluid phase in the cultures and, second, as a component of the standard 5:3:2 mixture.

A number of other media ingredients which are occasionally used in culturing were similarly examined as submitted to the laboratory. These ingredients included phenol red solutions, beef embryo extract, ascitic fluid, serum ultra filtrate, and a variety of synthetic media.

Besides defining the media ingredients as usable or not in terms of cell response, it was decided to analyze them relative to content of components having possible significance in culturing. Samples of plasma were accordingly examined for content of calcium and lipid and for their clot-forming properties. Embryo extract was analyzed for calcium, for nucleic acid phosphorus, and for thromboplastic activity; the sera were tested for content of calcium, lipid, and hemoglobin. The results are summarized in TABLES 2, 3, and 4. When,

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[*] See appendix.

[†] Concentrations of embryo extract are frequently expressed as percentage of whole embryo juice, EE_{50}, EE_{20}, *etc.* If, for example, chick embryos are put through a tissue homogenizer without diluent and the resulting tissue mash is centrifuged, the supernatant is referred to as EE_{100}. When an equal volume of balanced salt solution is included with the embryos the resulting extract is referred to as EE_{50} *etc.*

TABLE 1

SUMMARY OF TESTING SCHEDULES

Test object: explants of skeletal muscle from 14-day-old chick embryo

Commercial Media				Control Media		
Medium examined	Culture vessel	Test 1	Test 2	Medium examined	Test 1	Test 2
Plasma	Roller tubes Carrel flasks	50% plasma and b.s.s. only	33% plasma and Nutrient 5-3-2	Plasma	50% plasma and b.s.s. only	33% plasma and Nutrient 5-3-2
Embryo extract	Roller tubes	Plasma substrate 20% EE_{20} supernate	Plasma substrate and nutrient 5-3-2 with test EE_{20}	Extract	Plasma substrate and 20% EE_{20} supernate	Plasma substrate and nutrient 5-3-2
Balanced salt solutions (b.s.s.)	Roller tubes	12% plasma and b.s.s. only		Grey's sol.	12% plasma and b.s.s. only	
Serum Human Horse Sheep	Roller tubes	12% plasma and 30 % serum	Plasma substrate and nutrient 5-3-2 with test serum	Human cord serum	12% plasma and 30% serum	Plasma substrate and nutrient 5-3-2

This table summarizes the culture tests to which each media component was subjected. In the case of plasma, *e.g.*, the explants were placed in a clot consisting solely of plasma and balanced salt solution. It was expected that, under these conditions, any toxicity the plasma might possess would be made evident. A second test for plasma involved mixing it with 2 volumes of regular 5:3:2 nutrient. This procedure provides for rapid culture growth which might be expected to reveal any unusual tendency for the test clot to lyse.

In the case of each of the other materials, as with plasma, the tests were designed to place the culture in as high a concentration of the test material as seemed consistent with growth of the cells. Differences in rate of growth and migration were taken to reflect differences between commercial and control components.

TABLE 2
ANALYSES OF PLASMA

Properties Measured	Chicken Plasma	
	Commercial	Control
pH		
Mean value	9.0 ± 0.3	8.0 ± 0.3
No. of samples	14	5
Calcium, mg. %		
Mean value	9.9 ± 0.5	10.3 ± 1.0
No. of samples	19	6
Lipid, mg. %		
Mean value	320 ± 120	725 ± 200
No. of samples	19	7
Hemoglobin, g. %		
Mean value	0.02 ± 0	0.016 ± 0.005
No. of samples	19	7
Clotting time		
Mean value	4′20″	1′5″
No. of samples	19	5

This table records analyses of important properties of plasma. The *hydrogen ion* concentration of the commercial sample is abnormally high as a result of CO_2 depletion during lyophilization. It can be restored to near normal by placing the reconstituted plasma under 5 per cent CO_2 in air.

Calcium determinations were made according to a method described by Peters and Van Slyke[5] as a modification of the Halverson and Bergeim procedure.

The procedure of Bloor[2] was followed for all measurements of *lipid* content. It would appear that, in this respect, the commercial plasma was better than the control, possibly because the cockerels supplying the commercial material were deprived of food for a longer period before bleeding. Calculations of *hemoglobin* content were made from spectrophotometric absorption values. *Clotting times* are for lyophilized commercial plasma and freshly collected control plasma. The longer times required by the commercial product results from lyophilization, for when the commercial material was shipped to the laboratory in the liquid state, its clotting time was identical with that of laboratory-collected plasma. All clotting determinations were made by mixing equal volumes (0.5 ml.) of 50 per cent plasma (using Gey's solution as diluent) and EE_4 prepared in the laboratory as EE_{50} and diluted just before using. The time which elapsed between mixing and the development of a clot which would hold in an 8-mm. tube (*i.e.*, when tilted) was taken as the *clotting time*. Tubes were kept at 25° C. during the determination, and exactly the same procedure of mixing, *etc.* was employed for each tube. The embryo extract was compared with a standard solution of thrombin to make sure it had the usual thromboplastic activity.

under analysis, any particular sample of commercial media showed pronounced deviation from the control materials with respect to the above components, it was considered suspect and usually rejected, especially if the deviation found some reflection in the cultured cell tests. As these analytical data were collected and as corresponding culture tests were made, some attention was given to correlating the two. However, in the period of laboratory operation there was not sufficient time to explore suggestive results to the point of significant conclusions.

Following a period of several months, during which the laboratory and testing program were being organized, a total of 101 samples were given thorough study and, of these, 76 were found satisfactory and certified for distribution. In many instances, the samples submitted were experimental in the sense that the commercial house was examining some method of preparation or preservation. This fact accounts, in part, for the high percentage of rejections. Now that reliable procedures have been established, the incidence of substandard samples would be much lower.

The testing and analyses of media components were not the sole activities of the laboratory. As time permitted, studies were made of a variety of problems associated with the use and preparation of commercial media.

TABLE 3
ANALYSES OF SERA

Properties Measured	Horse Serum		Human Cord Serum	
	Commercial	Control	Commercial	Control
pH				
Mean value	6.1 ± 1.1	7.9 ± 0.3	8.8 ± 0.4	7.9 ± 0.1
No. of samples	6	4	3	5
Calcium, mg. %				
Mean value	8.7 ± 1.1	9.9 ± 1.5	8.5 ± 0.6	9.7 ± 0.5
No. of samples	6	4	6	3
Lipid, mg. %				
Mean value	545 ± 90	340 ± 75	250 ± 120	460 ± 50
No. of samples	6	4	6	5
Hemoglobin, g. %	(turbid)		(turbid)	
Mean value	0.07 ± 0.02	0.02 ± 0	0.08 ± 0.04	0.045 ± 0.01
No. of samples	6	4	6	6

The determinations summarized here were made by procedures identical with those used for plasma.

It was important, for instance, to obtain some measure of the stability of the lyophilized material under various conditions of storage. The experiments were limited, but they demonstrated no significant change in the properties of lyophilized plasma or embryo extract stored at room temperature for one month or at 4° C. over a period of one year. Data are not available for longer periods under these conditions. The reconstituted plasma was stable over two weeks at 4° C., but reconstituted extract changed detectably except when frozen or stored at −16° C. In this connection, an interesting fact came to light in that, under storage in dry ice (rubber capped tubes), there was a 30 per cent decrease in nucleic acid phosphorus. No attempt was made to account for this effect, but the measured decrease did not find any expression in parallel culture tests.

Other experiments were made to explore (a) differences in embryo extract derived from embryos of different ages and (b) differences resulting from different procedures for grinding up the embryos. The results are presented solely in terms of nucleic acid phosphorus. Calcium measurements were not reported as sufficiently reliable. By this criterion (*i.e.*, nucleic acid phosphorus), extract prepared with the aid of a Potter tissue homogenizer was 30 per cent better than that prepared by simply forcing the embryos through a platinum mesh in a syringe. In similar terms, extracts prepared from 8-, 10- and 12-day-old embryos by identical grinder procedures were compared. The degree of difference varied considerably among the three experiments performed, but the nucleic acid phosphorus was consistently higher in the extracts from 10- and 12-day-old embryos, and as much as 30 per cent higher in the latter. If this ingredient is regarded as contributing significantly to the growth-promoting properties of the embryo extract, then preparations from 10- or 12-day-old embryos should be considered superior.

Plasma, in the lyophilized form, created some problems for the attention of the laboratory. When reconstituted with distilled water, as required, it was found to clot very slowly if at all. It was noted that the pH was abnormally

TABLE 4

ANALYSES OF EMBRYO EXTRACTS

Properties measured	Embryo extract (EE_{50}) commercial	Embryo juice (EE_{100}) commercial	Embryo extract (EE_{50}) control
pH			
Mean values	7.2 ± 0.1	7.2 ± 0.6	7.3 ± 0.4
No. of samples	12	11	4
Calcium, mg. %			
Mean value	7.9 ± 3.6	6.3 ± 1.6	5.4 ± 1.0
No. of samples	15	19	4
Inorganic phosphate, mg. %			
Mean value	27.2 ± 10.1	21.6 ± 5.0	22.0 ± 5.3
No. of samples	15	19	4
Nucleic acid phosphorus, mg. %			
Mean value	6.1 ± 3.1	6.1 ± 1.5	10.0
No. of samples	15	19	2
Dry weight, g. %			
Mean value	3.05 ± 0.59	2.90 ± 0.53	2.64 ± 0.30
No. of samples	15	18	4
Thromboplastic activity			
Mean value	1′ 35″	1′ 35″	1′ 10″
No. of samples	15	16	4

Except for thromboplastic activity (which was done with EE_4), all determinations above were made on EE_{50}

This table presents a summary of analyses made on commercial and control embryo extracts.

The *calcium* determinations were made by procedures used for plasma and serum. *Inorganic phosphate* determinations were made according to Berenblum and Chain.[1] *Nucleic acid phosphorus* content of the extracts was measured from the ashed residue after extraction of inorganic phosphate. In the procedure a 0.5-ml. sample of embryo extract (EE_{50}) was extracted with 4.5 cc. of 10 per cent trichloroacetic at 0° C. for 30 minutes to remove inorganic phosphate. The sediment was then ashed with 0.25 ml. of perchloric acid plus 1 to 2 drops of H_2O_2. The mixture was evaporated to 0.3 ml., then neutralized with freshly prepared NaOH (1 drop of phenophthalein as indicator), and finally evaporated to 1.0 ml. 0.5 ml. of this was taken for analysis.

In making determinations, the following proportions of reagents were used: 0.05 ml., 10 N H_2SO_4 ; 0.25 ml., 5 per cent NH_4MO_4 finally prepared; 0.50 ml., neutral sample; 0.20 ml., H_2O; 1.0 ml., isobutyl alcohol; 2 ×1.0 ml., wash 1 N H_2SO_4 ; 1.5 ml., diluted reducing agent.

As standard for colorometric determinations a freshly prepared solution of KH_2PO_4 containing 998 γ P/ml. was used.

high, and this observation led to the realization that the vacuum conditions employed in the freeze-dry procedure were removing the CO_2. Experiments were therefore conducted which compared clotting times before and after displacing the air over the reconstituted material with a mixture of 5 per cent CO_2 in air. In every case, the clotting time was reduced to near normal by the CO_2 treatment.

Finally, as time permitted, some attention was directed toward a better method of measuring growth, *i.e.*, by analyzing for increase in nucleic acid phosphorus. Although the procedures used were as good as were then available and coincided in most respects with those employed by Davidson and Waymouth,[3, 4, 5] the growth curves plotting increase in nucleic acid phosphorus against time varied considerably from one test run to another, even though culture conditions, *etc.* were identical. This very limited experience supported the view that colony diameters might not, after all, be such a poor measure of tissue response to the materials and conditions of culturing.

This report has summarized the work of the first laboratory for certifying commercially prepared culture media. Besides providing guidance for the manufacturers, the project demonstrated that culture media of suitable quality could be processed, packaged, and distributed on a commercial basis. The costs of production were initially high, and this fact served to discourage many potential consumers. Difficulties in learning to use a new source of media as well as the unsuitableness of certain of the initial products for certain types of culture discouraged other customers. There has continued to be, however, a small but steady demand for the various media ingredients which have become available. Latest reports from the major producers indicate that the recent upsurge in interest in mass culturing for the propagation of viruses has brought about a very substantial increase in demand for these products. This factor is important, for it will enable the commercial houses to prepare larger batches of material and thus reduce costs and achieve a greater degree of uniformity and standardization.

Appendix

Detailed culture directions were prepared and exactly followed for each individual medium component that was examined. These directions served to keep the testing constant over the period during which the laboratory functioned and made early and later measurements and observations comparable. Those for the testing of embryo extract are presented here only to illustrate the detail involved and the painstaking effort made to achieve uniformity. Equal attention to procedural detail was given to the testing of all commercial materials.

Culture procedures for testing of embryo extract: (1) Reconstitute lyophilized extract (5.0 ml. pkg.) with 4.5 ml. of triple distilled water. If the package content is labeled as EE_{50}* dilute reconstituted material with 1.5 volumes of Gey's balanced salt solution to give EE_{20}; (2) Thaw out control extract (EE_{50}) and dilute with 1.5 volumes of Gey's b.s.s. to give EE_{20}; (3) Centrifuge both control and commercial extracts so diluted for 10 minutes at 1000 R.P.M. and remove the supernatant. This is the EE_{20} to be tested; (4) Dilute each of the above supernatants (EE_{20}) with 4 parts of Gey's solution to give so-called 20 per cent extract (actually EE_4) which is to be tested straight on cells; (5) Prepare complete nutrient medium by mixing: 5 vols. Gey's solution; 3 vols. human cord serum; 2 vols. EE_{20}. [In one, use control (laboratory prepared) extract and, in the other, use commercial extract]. (6) Cut up tissue, *i.e.*, skeletal muscle from thigh of 14-day-old chick embryo, and place 2 mm. explants in small quantity (0.1 ml.) of test material in petri dish. Use separate dishes for each test material; (7) Add 0.1 cc. of chick plasma to clean roller tubes (18 × 150 mm.) and spread evenly over surface of lower part of tube. Place 5 explants on side of upper half of tube, withdraw surplus fluid from explants and then place them in a row in plasma-coated part of tube. Place tube in position to drain toward bottom and then withdraw excess fluid. Leave tube in horizontal position for 10 minutes for clotting and then stopper and place in incubator at 37.5°C. in roller drum for 2 hours; (8) After 2 hours accumulated fluid should be withdrawn and test solutions added as follows, each to three tubes:

(a) Control—0.5 ml. Gey's solution containing phenol red 0.01 mgm./liter.
(b) 0.5 ml. of 20 per cent control extract (EE_4).
(c) 0.5 ml. of 20 per cent commercial extract.
(d) 0.5 ml. of 5:3:2 nutrient medium with control extract.
(e) 0.5 ml. of 5:3:2 nutrient medium with commercial extract.

Phenol red to the extent of 0.01 mgm./liter is included in each culture. If the pH is above 7.4, the tubes should be gassed with 5 per cent CO_2 in air. Incubate for 4 days at 37.5° C. and then make measurements and observations.

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GROWTH OF CELLS IN AGITATED FLUID MEDIUM*

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Introduction

One of the difficulties arising from the use of tissue cultures in physiological studies has been the accurate evaluation of growth under experimental conditions. The methods which have been developed, in most cases, have been confined to a particular type of measurement which gives an indirect measure of the actual number of cells present at any one time. When compact cell colonies are formed, the determination of cell number may be difficult or impossible, and the evaluation of the conditions of the environment to which each individual cell of the colony is exposed often cannot be determined. The cells on the edge of the colony are exposed directly to the nutrient fluid, while those in the center are exposed partially or exclusively to living or handicapped cells.

In the method to be reported here, cells which do not form colonies and are maintained in suspension have been used. The cell suspension is kept agitating so that the medium is mixed continually and all of the cells are exposed to uniform environmental conditions. Samples of the suspension are removed for cell counts.

Since Harrison[10, 11] developed the first method for growing animal tissues *in vitro*, it was thought by many investigators that a supporting framework consisting of a substance such as fibrin was necessary for optimum growth of tissues in culture.[6, 7, 8] Since then, it has been found that, in the case of many cell types, good growth occurs on a glass surface in a liquid medium.[9, 12] It has been found now that cells can be grown without being permanently attached to a surface. This method was first reported at the meetings of the American Association for Cancer Research held in April 1953.

Materials and Methods

The strain of cells used in these experiments was derived from de Bruyn's mouse lymphosarcoma MB(T86157).[2, 3, 4, 5] In de Bruyn's primary tissue cultures of this tumor, a symbiotic relationship appeared to be established between malignant lymphoblasts and the normal mesenchyme. These tumor cultures had an abundant growth of fibroblasts, with many lymphoblasts and a few macrophages. Continuous cultures of this tumor, which can be maintained indefinitely, have only lymphoblasts and fibroblasts. Twenty-three days after the explantation of the tumor, the fibroblasts in one series of cultures had become swollen and granular. Following transfer of one of these cultures, the fibroblasts showed greatly diminished migration, but the lymphoblasts continued to grow luxuriantly. From 49 to 61 days after explantation, the fibroblasts from the above-mentioned culture became very few in number.

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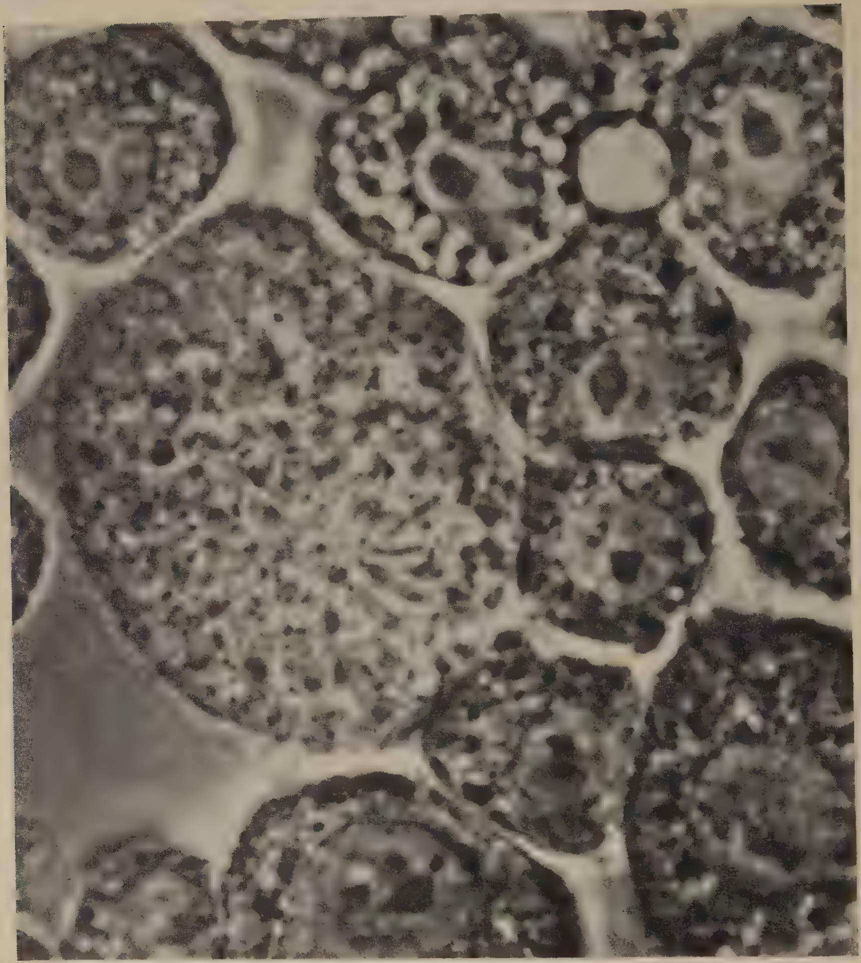

FIGURE 1. Appearance of the strain MB III in a slide culture, photographed at 500 × with phase optics. The variation in size of cells is typical. The largest cell is in prophase.

This culture, which had been grown on a plasma clot, was then transferred to a fluid medium and the lymphoblasts continued to grow on the glass of the tube in the absence of the fibroblasts. This pure lymphoblastic strain, designated MB III (formerly MB 13), was continued and showed a gradual loss of malignancy when it was transferred into originally tumor-susceptible mice. After 157 days of cultivation, the lymphoblastic strain failed to produce tumors. Since then (1948), the strain has been cultivated in this laboratory in roller tubes and continues to produce no tumors in tumor-susceptible mice, whereas the original malignant mixed cultures, containing lymphoblasts and fibroblasts, continue to produce tumors. FIGURE 1 shows the appearance of the strain MB III in a slide culture prepared from the roller tube strain.

TABLE 1

Gey's balanced salt solution (BSS)*	16%
Beef embryo extract	16%
Human placental cord serum	67%
Penicillin	100 units per ml.

* Type II for equilibration with air.
Constituents of medium for stock roller tube cultures of MB III.

The medium in which the strain has been cultivated includes the constituents shown in TABLE 1.

In the roller tubes, the cells grow rapidly, most of them adhering to the glass surface of the tube. A four-day-old roller tube culture has an even layer of cells over the surface of the part of the tube which is exposed to the medium. The cells are transferred to new tubes twice a week by being washed from the glass into the fluid. Half of this cell-containing fluid is then added to a fresh tube containing the above medium. In these cultures, free-floating cells are found which give the medium a turbid appearance. The free-floating cells are viable and can be subcultured. With slight agitation, large numbers of cells are freed from the glass. For these reasons, it was decided to use this strain for development of the suspended cell technic. FIGURE 2 shows the appearance of a two-day-old culture of this strain, MB III, in a roller tube, photographed at low power (100X).

When a cell suspension of MB III is allowed to remain quiet, the cells become adherent to the sides of the tube, causing a decrease in suspended cells of about 5 per cent in five minutes. An apparatus was designed which would shake the culture tube continuously and wash the nutrient medium over the entire inner surface of the tube to prevent the cells from settling down and accumulating in quiet areas. The new type of culture tube which has been devised and named "tumble tube" is shown in FIGURE 4. The tube is a 13 mm. × 50 mm. Pyrex test tube. The tube, containing the medium and the cells to be cultured, is placed in a clamp, and a Teflon pad is fitted tightly against the open end by means of a screw shown in FIGURE 4 (G). In order to have a good seal with the Teflon pad, the open end of the tube must have a finely ground and fire-polished rim. The tube, screwed tightly into its clamp, is fastened onto the face of a wheel turning at 38 rpm, approximately once every two seconds, and located in a 37° incubator. The wheel keeps the tube constantly in motion and the cell suspension washing over the entire inner surface of the culture tube. At this speed, there is no frothing of the medium, as was demonstrated with more rapid speeds, and the cells are prevented from settling down. Several preliminary trials with other types of less practical tubes gave satisfactory results. One type was similar to the present tumble tube, but was sealed by being drawn out in a flame.

The tumble tube cultures were set up as follows. Strain MB III cells and the fluid were removed from the stock roller tube by gently washing and scraping the cells from the walls of the tubes with a bent-tip tissue culture pipette. These cells were washed into the fluid medium at the bottom of the tube to make a cell suspension. The cell suspension was transferred either

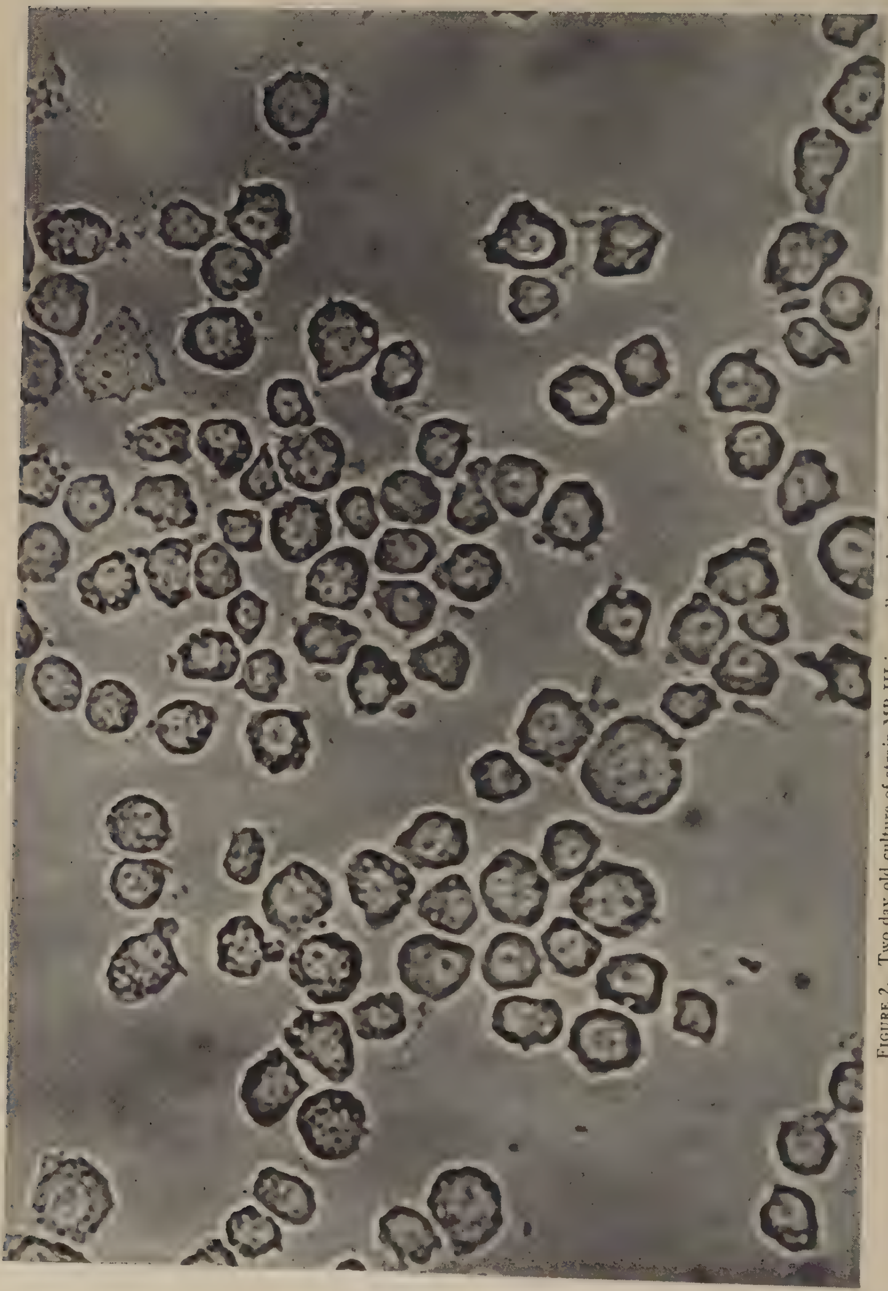

FIGURE 2. Two day old culture of strain MB III in a roller tube, photographed at low power (100 ×).

directly to fresh medium in the tumble tubes or was centrifuged and the fluid decanted. In the latter case, the cells were resuspended in fresh medium or in a mixture of old medium and fresh medium. Each tumble tube contained a total of 1 ml. of cells and medium, leaving an air space in the tube of about 3.4 ml.

Growth of the cell population was determined by counting the cells with a hemocytometer at the start, and after three days, or at various intervals during an experiment. Chilling did not alter the counts, therefore several tubes were removed from the wheel simultaneously and put into the refrigerator until counts could be made. The results were subjected to statistical analyses.

For observation of the cells, the sealed tumble tubes in the clamp assembly were removed from the wheel. These observations were made with an inverted microscope, which brings the cells into focus from below. It could be seen quite clearly that a large majority of the cells were in suspension, since they could not be kept in focus while they were in the process of settling out. If the tube were tipped slightly, they would roll over one another. It could be seen also that they were roughly spherical. After a few minutes without disturbance, many of the cells became motile and showed typical lymphoid movement.[16] The cells often occurred in clusters of up to 20 cells, although, in most cases, the majority of cells occurred singly. The clusters were not permanent, for the proportion of cells in clusters increased when the tube remained quiet and, when the tubes were opened and thoroughly mixed, prior to counting, the clusters disappeared. FIGURE 3 shows the appearance of a two-day tumble tube culture, photographed at low power (100 times) with the inverted microscope, after the cells had been allowed to settle for several minutes. The cells that are not in focus have not yet settled down.

The tendency of the cells to stick to any glass surface with which they come into contact makes accurate pipetting of the cell suspension difficult. We found it necessary to use a standard mixing procedure in order to make accurate and reproducible dilutions. Some erratic results in the earlier experiments were traceable to inadequate mixing of the suspension and variable pipetting technique.

In order to determine the limitations of this new method we have explored various experimental procedures. In no case are the experiments exhaustive or entirely conclusive. It is the purpose of this report to present the results obtained and to show how the cells may be expected to behave under a variety of conditions.

Evaluation of Growth

FIGURE 5 shows increase in cell number over a period of one week. The points represent the means for two tubes, eight separate counts per tube. The vertical lines represent the standard deviations for the 16 counts. The values on the ordinate are expressed as millions of cells per ml. or per tube, since each tube holds one ml. Note that, in this case, we started with approximately 800,000 cells per tube and that, after five days, the number had increased to 3,300,000 cells, a fourfold increase.

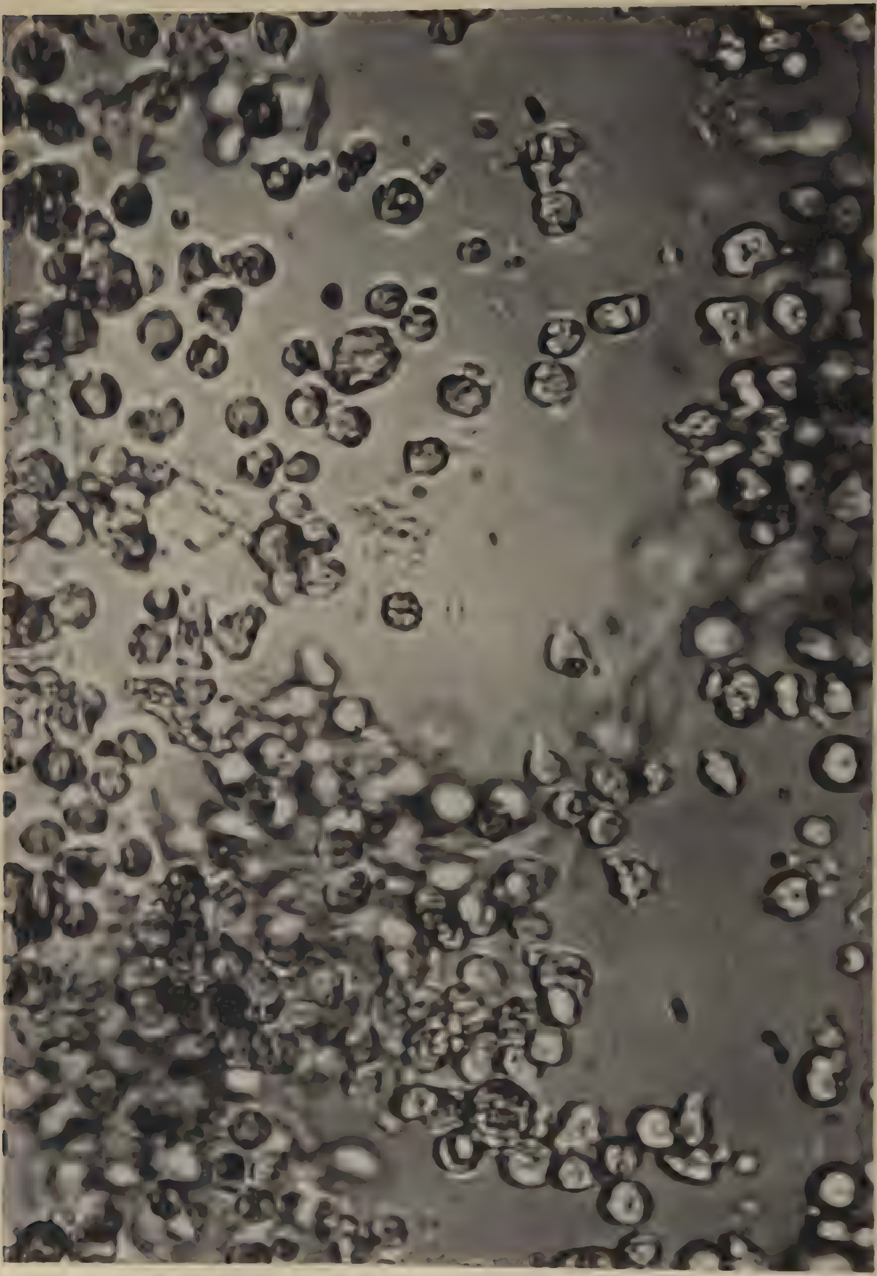

FIGURE 3. Two day old culture of strain MB III in a tumble tube, photographed at low power (100 ×).

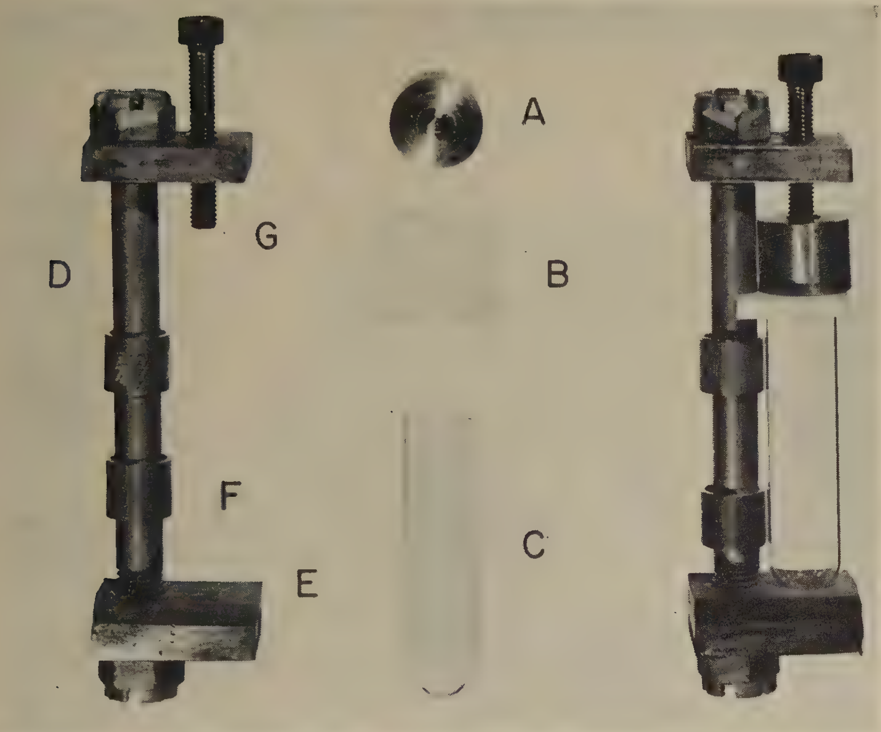

FIGURE 4. Tumble tube and clamp assembly. On the right is the complete assembly and on the left are the separate parts. (A) backing for Teflon cap; (B) teflon cap; (C) culture tube; (D) frame; (E) rubber pad; (F) collars to aid in alignment of tube; (G) screw for tightening cap.

In most experiments, a three-day growth period was used as the standard for determining the effect of various conditions and agents on the growth of the cells. This period was adopted because, in some instances, growth occurred very rapidly and reached its peak between three and four days. After that time, the cultures degenerated and cell identification became difficult because of the increasing proportion of dead and degenerating cells.

Careful examination of the tumble tube cultures at intervals, during several days' incubation, showed that there were some cells adhering to a proteinogenous precipitate on the glass. The precipitate reached its greatest volume at 24 hours. Only a very small amount remained after 48 hours, and none was visible after 72 hours.

An experiment was set up to determine the relation of the number of cells in suspension to the number of cells adherent to precipitate or attached directly on the glass. A group of eight tumble tube cultures was used. Two tubes were removed each day for four days. From one of these tubes, the medium with the suspended cells was carefully removed with a tissue-culture pipette without disturbing the precipitate on the glass. This suspension was put into another tube and set aside for counting. One ml. of BSS was then

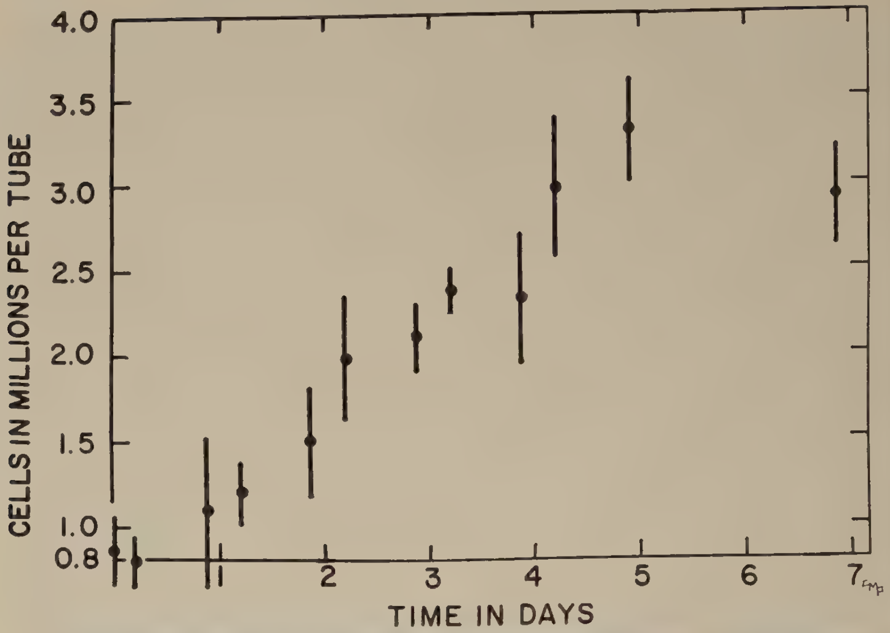

FIGURE 5. Increase in number of MB III cells in tumble tubes over a period of one week. The points represent the means for two tubes, eight separate counts per tube. The vertical lines represent the standard deviations for the 16 counts.

added to the tube containing the precipitate. The precipitate was scraped off and thoroughly mixed into the BSS. This suspension, then, contained those cells which had been attached to the precipitate or on the glass. Cell counts were made of both the removed cell suspension and the cells in the BSS. A third count was made of the other tumble tube culture, a control, in which the medium and the precipitate were thoroughly mixed together. FIGURE 6 shows the results of this experiment. After 24 hours, 36 per cent of the total number of cells were not in suspension. From 48 to 96 hours, the number not in suspension was 3.5 per cent to 1 per cent, within the experimental error. The experiment showed that counts of the cell suspension at three days gave reliable data on the number of cells present in suspension, since the number of cells on the surface of the tube was insignificant.

Effect of Population Size

In order to determine whether slight variations in the number of cells present at the start of an experiment might account for the relatively large differences in magnitude of growth from one control group to another, an experiment was set up in which the starting number of cells per tube was varied from 700,000 cells to 1,200,000. In this experiment, it was necessary to pool the cell suspensions from a large number of roller tubes, determine the cell population of the pooled suspension with hemocytometer counts, and pipette appropriate quantities of the suspension into five test tubes to obtain specific numbers of cells in

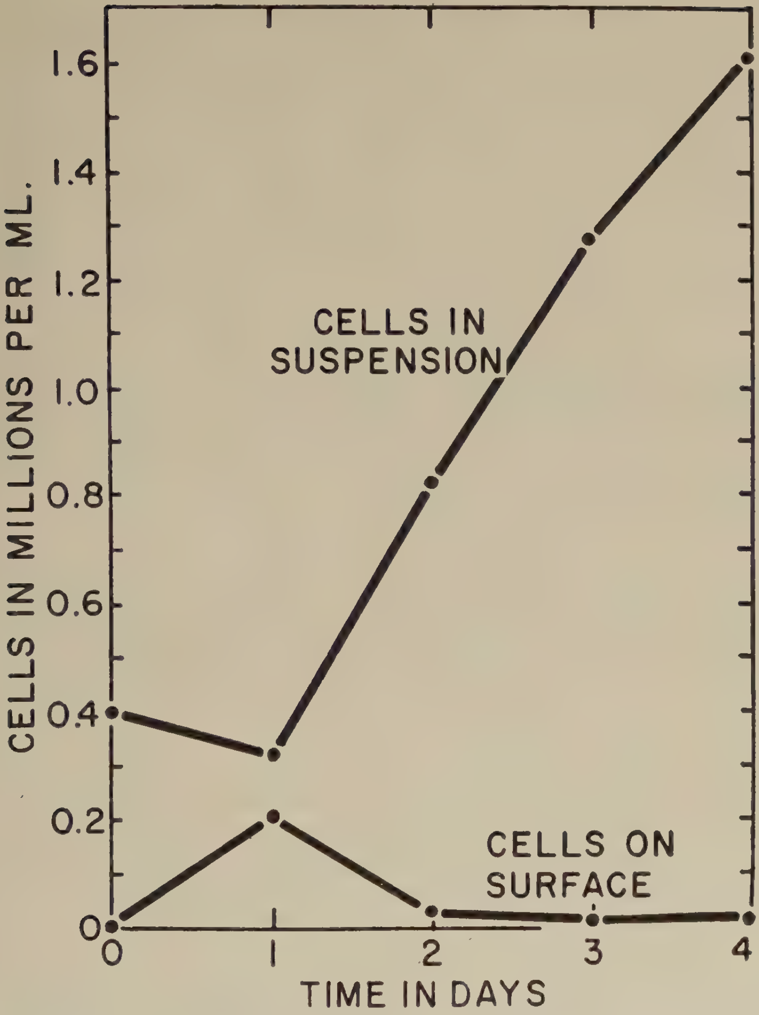

FIGURE 6. Four day growth of MB III cells in tumble tubes showing the number of cells in suspension compared to the number on the walls of the tube.

these tubes. The five tubes were then centrifuged and the old medium decanted. Constant amounts of fresh medium were added and the cells were resuspended. The contents of the five test tubes were pipetted into five groups of tumble tubes, four tumble tubes per group. The population of the remaining few milliliters of the five cell suspensions was determined and considered as the starting population of the five groups. These groups contained the following cell populations respectively: 1.22, 1.08, 1.02, 0.968, 0.764 million.

After three days, all of the tumble tubes were removed and the population determined. TABLE 2 shows the results of the experiment. When the cell population at the start of the experiment was low, Group I, there was a greater

TABLE 2

Group No.	No. of tubes	Initial population. Millions of cells per tube ± S.E. of the mean	Population after 3 days. Millions of cells per tube ± S.E. of the mean
I	4	0.764 ± 0.020	1.77 ± 0.041
II	4	0.968 ± 0.057	2.03 ± 0.058
III	4	1.02 ± 0.044	2.30 ± 0.069
IV	4	1.09 ± 0.040	2.25 ± 0.086
V	4	1.22 ± 0.053	2.43 ± 0.073

The effect of the size of the population on three-day growth. The population size is expressed as number of cells per tube ± the standard error of the mean. The lower population, Group I, produced a greater proportional increase than the higher population, Group V.

proportional increase in number of cells, than when the cell population at the start of the experiment was high, Group V. This difference in proportional increase was not great enough to account for the relatively large differences encountered from the control group of one experiment to the control group of another experiment. When the cell population was very large, 11,000,000, no increase in cell number could be detected.

Composition of the Medium

In the experiments in which the medium itself was not under investigation, the composition of the fresh medium was the same as that used on the stock roller tubes (TABLE 1). Later on, when other aspects of the method had been improved, several variations in the composition of the medium were tried. The per cent serum and BSS were varied, while the per cent embryo extract was kept constant at 10 per cent. TABLE 3 shows the cell number obtained after three days' growth in 40 per cent, 60 per cent, and 80 serum, each with 10 per cent embryo extract. The probability that the difference found between 40 per cent serum and 60 per cent serum was owing to chance is less than 0.01. The probability that the difference between 60 per cent serum and 80 per cent serum was owing to chance lies between 0.05 and 0.10. It was concluded that 80 per cent serum produced a greater three-day increase than 40 per cent or 60 per cent. A subsequent experiment has shown that 90 per cent serum produced a greater cell number than 80 per cent, but that 100 per cent serum, that is, no embryo extract or BSS, produced poor growth.

TABLE 3

Group No.	No. of tubes	Components of the medium in per cent			Initial population. Millions of cells per tube ± S.E. mean	Population after 3 days. Millions of cells per tube ± S.E. mean
		BSS	Embryo extract	Cord serum		
I	3	50	10	40	0.874 ± 0.031	1.56 ± 0.040
II	3	30	10	60	0.874 ± 0.031	1.81 ± 0.069
III	3	10	10	80	0.874 ± 0.031	1.97 ± 0.057

Effect of the cord serum. The initial population was determined by counting a cell suspension, adding it to the three media and multiplying the count of the original suspension by the dilution factor. A greater increase in cell number was obtained with higher per cents of cord serum.

TABLE 4

Group No.	No. of tubes	Components of the medium in per cent			Initial population. Millions of cells per tube ± S.E. of the mean	Population after 3 days. Millions of cells per tube ± S.E. of the mean.
		BSS	Embryo extract	Cord serum		
I	3	15	5	80	1.25 ± 0.056	3.01 ± 0.086
II	3	10	10	80	1.25 ± 0.056	3.11 ± 0.140
III	3	0	20	80	1.25 ± 0.056	2.95 ± 0.080

Effect of embryo extract. No differences in three-day growth could be detected with these per cents of embryo extract.

In the next experiment, the concentration of serum was kept constant at 80 per cent, while the embryo extract and BSS were varied. TABLE 4 shows the values obtained at the start and after three days' growth. The differences found were not considered significant (P = 0.1–0.5).

From these experiments, it was concluded that, for maximum growth, the medium should contain from 80 per cent to less than 100 per cent serum and need not contain more than 5 per cent embryo extract.

The penicillin which was included in the medium was found to have no effect on the rate of growth, as TABLE 5 shows. This table, which includes two separate experiments, also serves to show the precision of the method. The first experiment gave results with a high degree of precision. The second experiment gave less precision, but the difference between the control and the penicillin-treated tubes was not significant.

The Cell Conditioned Medium

It was mentioned previously that the number of cells present in the tubes at the beginning of an experiment was usually about 1,000,000, but the number varied from about 0.500,000 to about 1,500,000. No attempt was made to maintain the number constant from one experiment to another because it would have been necessary to subject the cells to additional manipulation which, as experience had shown, frequently introduced new sources of error.

TABLE 5

Group No.	No. of tubes	Treatment	Initial population. Millions of cells per tube ± S.E. mean	Population after 3 days. Millions of cells per tube ± S.E. mean
I	4	Control	1.43	3.98
II	4	Penicillin (100 units/ml.)	1.43	3.98
I	3	Control	1.03 ± 0.064	3.39 ± 0.088
II	3	Penicillin (100 units/ml.)	1.03 ± 0.064	2.90 ± 0.077

Two separate experiments showing the effect of penicillin. These experiments demonstrate the precision of the method. The first (upper) experiment shows, with precision, that no difference could be found between penicillin treated and control tubes. The second experiment (lower) gave less precise results, but shows again that penicillin had no detectable effect (P = 0.5).

TABLE 6

Group No.	No. of tubes	Treatment	Initial population. Millions of cells per tube ± S.E. mean	Population after 3 days. Millions of cells per tube ± S.E. mean
I	8	Control	1.03 ± 0.064	3.39 ± 0.088
II	8	Washed	1.03 ± 0.064	2.90 ± 0.077

Effect of washing on subsequent growth of the MB III cells. There was significantly less growth in the cultures containing washed cells than in the unwashed controls ($P < 0.01$).

The cells could not be subjected to simple dilution without also diluting the old medium in which they were suspended. This old medium was an important factor in regulating the growth rate.

It was reported by de Bruyn[3] that when these lymphoblastic cells were transferred from one roller tube to another, growth of the cells following transfer was greater when the medium consisted of at least one half of old medium plus one half new medium rather than entirely new medium. Since we wished to study the effect of various components of the medium we attempted to grow these cells in entirely new medium. The procedure was as follows: a cell suspension from several roller tube cultures, obtained as previously described, was centrifuged at 2500 rpm (1500 × gravity) for 10 minutes and the supernatant decanted with a pipette. The cells were then resuspended in 10 per cent serum in BSS and recentrifuged. This procedure was repeated, and then these washed cells were resuspended in fresh medium and pipetted into eight tumble tubes. Eight control tubes were set up also. TABLE 6 shows the increase in cell number after three days. Those cells which were washed free of the old medium had significantly ($P < 0.01$) less growth than the control, those containing 30 per cent old medium. There was some uncertainty as to whether the loss in growth capacity was attributable directly to the lack of old medium or whether the washing procedure was injurious to the cells. Another experiment was set up in which the amount of old medium was varied from a trace to 50 per cent (TABLE 7). In this case, the whole cell suspension was centrifuged once in order to decant the old medium. The cells were not resuspended in the 10 per cent serum. Consequently, the "trace" means only that no old medium was added back after centrifugation. There was undoubtedly some old medium present. TABLE 8 shows that variations from a trace to 30 per cent old medium made no difference in the rate of growth, but

TABLE 7

Group No.	No. of tubes	Per cent old medium	Initial population. Millions of cells per tube ± S.E. mean	Population after 3 days. Millions of cells per tube ± S.E. mean.
I	4	"trace"	1.56 ± 0.038	3.34 ± 0.087
II	4	10	1.56 ± 0.038	3.31 ± 0.116
III	4	30	1.56 ± 0.038	3.23 ± 0.082
IV	4	50	1.56 ± 0.038	4.12 ± 0.065

Effect of cell-conditioned medium. No promotion of growth occurred unless the medium consisted of 50 per cent old medium.

TABLE 8

Group No.	No. of tubes	Per cent old medium	Initial population. Millions of cells per tube ± S.E. mean.	Population after 3 days. Millions of cells per tube ± S.E. mean.
I	3	"trace"	1.19 ± 0.012	2.86 ± 0.089
II	3	10	1.19 ± 0.012	2.99 ± 0.135
III	3	25	1.19 ± 0.012	2.96 ± 0.096
IV	3	50	1.19 ± 0.012	2.90 ± 0.104
V	3	70	1.19 ± 0.012	3.30 ± 0.157

Same as TABLE 7, but no promotion occurred unless the medium contained 70 per cent old medium.

that 50 per cent had a definite growth-promoting effect. The experiment was repeated, and this time the final suspension contained a trace and the following per cents of old medium: 10 per cent, 25 per cent, 50 per cent, and 70 per cent, as shown in TABLE 8. In this second experiment, 70 per cent old medium produced greater growth than all the tubes containing lower per cents. It was concluded from the last three experiments that growth will proceed at a lower rate when the old medium is present in amounts up to 50 per cent but that a definite promotion of growth occurs when the old medium is included in amounts from 50 per cent to 70 per cent. The old medium varies considerably in spite of our standard procedure and the growth-promoting effect may not be observed except at higher concentrations (above 50 per cent). This conclusion confirms de Bruyn's reported observations.

It can be seen from examination of TABLES 2, 7, and 8 that a dilution of a cell suspension would have a twofold effect on subsequent growth: (1) a dilution of the cell number which, within limits, increases the proportional growth of the cell population; and (2) a dilution of the old medium, which, within limits, decreases the proportional growth of the cell population. It was therefore decided that, for the time being, it would simplify the experiments if no attempt were made to start each experiment with the same number of cells. For convenience in manipulation, the tumble tubes were usually started with 30 per cent cells suspended in old medium and 70 per cent fresh medium. The treatment of the roller tubes prior to transfer to the tumble tubes followed a standard procedure and the cell population was maintained fairly constant.

Hyaluronidase and Trypsin*

After several hours in the tumble tubes, the cells tended to form clusters of 5 to 20 cells. A certain amount of proteinogenous precipitate formed also. In order to prevent both the cluster formation and the precipitation of some proteins during shaking, hyaluronidase was added to the cultures. The final concentration of hyaluronidase was 0.1 per cent. FIGURE 7 shows the effect of the enzyme on growth. The greater number of cells in the hyaluronidase-treated cultures is a real difference, that is, it is not attributable just to the fact that there were fewer clusters because, in any case, when the tubes were opened for counting, the suspension was mixed with a pipette and the clusters

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* The authors are grateful to Doctor Wilton R. Earle for supplying the first lot of hyaluronidase. They are grateful also to Doctor Lawrence L. Lachat, Biochemical Market Development, Armour and Company, for a subsequent lot.

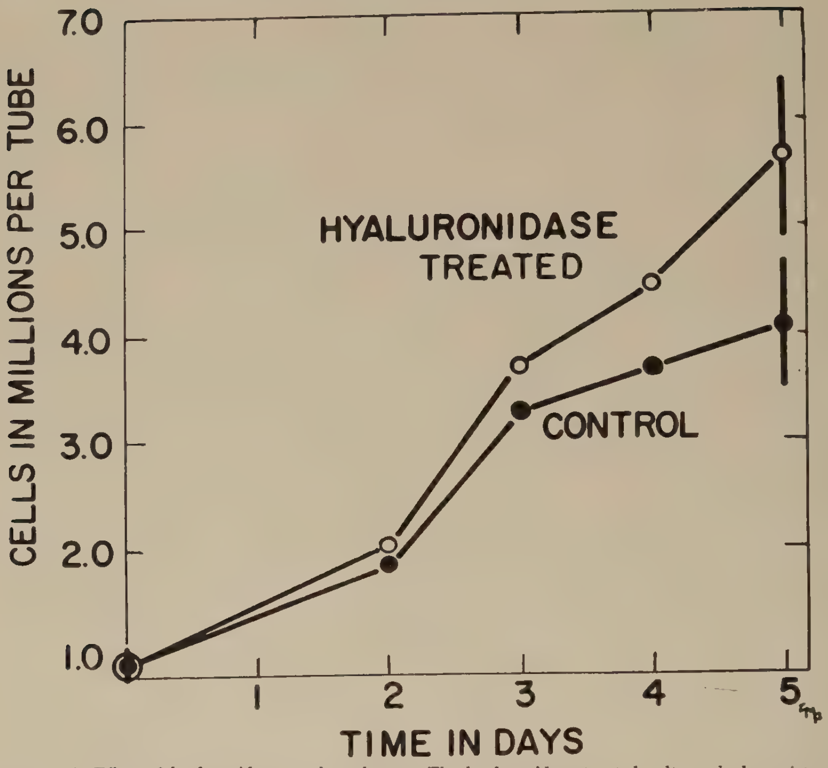

FIGURE 7. Effect of hyaluronidase on the cultures. The hyaluronidase treated cultures had consistently greater growth over the five day period. The standard deviations for the final values are shown as vertical lines.

were broken up. It was noted also that the hyaluronidase-treated cultures had little or no visible precipitate. It is not known whether the greater growth in the presence of hyaluronidase was owing directly to the hyaluronidase or whether it was attributable to one or several indirect factors. The fact that promotion of growth is correlated with the lessening in the amount of precipitate present indicates that the hyaluronidase may act by making available nutrient material which otherwise would be tied up in precipitate. On the other hand, the hyaluronidase preparation is relatively impure and may contain other active substances.

The addition of trypsin did not have the same effect as hyaluronidase. It was not effective in promoting growth or in digesting the precipitate. Digestion of the precipitate occurred only at higher concentrations which were injurious to the cells.

Experiment with Continuous Cultures

Several attempts have been made to maintain these cells in the tumble tubes for longer than one week. Examination of the growth curve (FIGURE 5), shows that the greatest growth rate is at two to three days. In the routine maintainance of MB III, roller tube transfers are made every three or four days. It was thought, at first, that transfer at three or four days would be sufficient with cultures in the tumble tubes, but all attempts were unsuccessful. The cultures died out after about two weeks.

An experiment was devised to test the ability of these cells to survive for varying lengths of time in the tumble tubes. Eighteen tubes were set up in the standard way. They were transferred to new tubes in groups of two, one member of each group diluted to one half with fresh medium at each transfer and the other member diluted to one third at each transfer. The serial transfer schedules in day intervals was as follows: 2–2–2, 2–3–2, 2–4–2, 3–2–3, 3–3–3, 3–4–3, 4–2–4, 4–3–4, 4–4–4. At the final transfer of each group, the cells were put back into roller tubes and observed. In this experimental period, six to twelve days, the only cells to survive the transfers were those transferred according to the first sequence: 2–2–2, or every two days. All others, those that remained in the tumble tubes for longer than two days, failed to survive the transfers. The information gained from this experiment led to another attempt to maintain the cells in the tumble tubes for a long period of time and through many transfers. This time, the transfers were made every two days. The cultures continued to grow for 42 days and died out when transfer on the second day was omitted. Another group was maintained for 54 days.

Discussion

It is the intention of the authors to use this method for the study of some of the nutritional factors necessary for growth of cells. Some preliminary experiments on the usefulness of the method in such studies have given very encouraging results. For example, growth of the strain MB III has been tried with various concentrations of the mineral salts in the BSS and it has been found that slight variations produce detectable differences in growth rate.

Other cell strains have been tried; *viz.*, HeLa, a human epidermoid carcinoma, and D-1 Re, a human chondromyxosarcoma. The epidermoid cells are ordinarily held in place by connective tissue and are not found in suspension, as lymphoid tissue often is. The epidermoid tissue grows well in the roller tubes forming a very loose tissue with many cells existing singly. In the tumble tubes, these cells do not remain in suspension but collect in loose masses of tissue on the side of the tube. They grow well there in spite of the rapid movement of the tube and fluid. Hyaluronidase and trypsin have failed to release the cells from the glass.

The chondromyxosarcoma showed good growth in suspension and has been maintained through several transfers. No quantitative studies have been made on these two strains.

Summary

The advantages of a method for growing cells in suspension were evaluated with a strain of nonmalignant lymphoblasts (MB III) derived from de Bruyn's mouse lymphosarcoma T86157.

A special apparatus was designed to rotate glass culture tubes end over end, providing continual agitation of cells and medium. It was found that a speed of 38 rpm gave no frothing and provided adequate agitation for good cell dispersion. A simple tube assembly was developed to hold the tube and a Teflon cap and to permit microscopic observation of the growing cultures. The tube has been designated "tumble tube."

Cells grown for prolonged periods in agitated media were not permanently altered. In two preliminary trials, agitated cell cultures were maintained for 40 and 50 days respectively when transfers were made every two days.

MB III cells grown by this method showed a fourfold increase in five days in a medium containing approximately 16 per cent beef embryo extract, and 67 per cent human placental cord serum.

Since the cells have a tendency to stick to each other or to the glass, the effect of agitation on cell dispersion was studied. After one day's agitation, 64 per cent of the cells were in suspension. After two to four days' growth and agitation, 95 per cent of the cells were in suspension. The addition of hyaluronidase caused an increase in cell dispersion and an increased growth rate.

The size of the initial population was important in determining the growth response. A population of approximately 1,000,000 cells gave a good growth.

The tumbling tube method permits an evaluation of growth response with changes in the serum concentration of the order of 10 per cent.

Washed cells, used as an inoculum in new medium, do not give a good growth response. The addition of 50 per cent or more of old medium (cell conditioned medium) gives greater growth.

The experiments reported here demonstrate the feasibility of growing cells in continuous suspension. The limitations of the method and some of the factors effecting the growth of MB III cells have been reported.

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Discussion of the Paper

Doctor G. M. Mateyko (*Washington Square College of Arts and Science, New York University, New York, N. Y.*): Methods have been mentioned for separating the cells of masses of tissues preparatory to explantation, or cells of established tissue cultures. These methods are mechanical agitation and the use of enzymes such as hyaluronidase and trypsin. It would be of interest to know which of the two enzymes is more desirable, since not only do they operate at different concentrations and pH optima, but on totally different substrates. Hyaluronidase acts to hydrolyze hyaluronic acid, a polysaccharidelike substance. This enzyme, it has been demonstrated, plays a role in the denuding of ova of surrounding cells,[1] and softens the intercellular cement of the connective tissue of capillaries.[2] On the other hand, it is well known that trypsin does not attact native proteins. It may be that cell separation is effected by the proteolytic effect on dead cells, although the ground substance of certain cells has been shown to be removed by this proteinase more rapidly than by hyaluronidase.[3] Certainly the nature of the intercellular material should be indicated wherever enzyme preparations are used, and it should determine the choice of enzyme. The possibility of injuring membrane should also be considered.

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THE APPLICATION OF MAMMALIAN CELLS IN CONTINUOUS CULTURE FOR ASSAYS IN VIROLOGY*

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Introduction

Stable cell strains of known viral range and rapid response to viral infection facilitate both theoretical and practical laboratory studies. For example, mammalian cells as stable strains in continuous culture have been employed in our laboratory since 1951 for assays in virology, with the result that cell cultures are used as routine instead of animals as indicators of viral activity and of the inhibitory effect of specific antibody. Moreover, this approach has proved satisfactory for measurements of the effects upon cellular metabolism of virus, antimetabolites, and chemicals. Many viruses exert a cytopathogenic effect which can be recorded directly stepwise by light microscopy, by phase-contrast microscopy, or by time-lapse cinephotomicrography. Of the four stable strains of cells used for viral assays, a strain of human malignant epithelial cell (strain HeLa, Gey) derived from an epidermoid carcinoma of the cervix[1] proved superior. In our laboratory this cell strain accordingly replaced explants of human and monkey tissues by satisfying the obvious need for a highly susceptible strain of human cell capable of yielding poliomyelitis virus[2, 3] and other viruses[4-9] in quantity, in from one to several days, and by its ready adaptability to the mass production of replicate cellular cultures. Moreover, studies have demonstrated that HeLa cells on glass provide a satisfactory host-cell virus system (a) for comparative metabolic studies of virus-infected and noninfected cells, and (b) in practical laboratory diagnostic tests for the isolation of poliomyelitis virus, for the specific allocation of field strains by immunologic type and for antibody assays. Accordingly, this paper in its presentation of evidence for the application of mammalian cells in continuous culture for assays in virology cites, by example, our experience with strain HeLa cells. A brief description is given (a) of the methods developed for the mass production of cells in continuous culture, and (b) of the procedures which are being employed for virus studies. A more complete description and details are available elsewhere.[3] References are made for comparative purposes to the experiences and results from the other laboratories that have employed stable cell strains for similar purposes.

Production of Strain HeLa Cells for Assays

Strain HeLa cells, like other animal cells, can be transferred from culture to culture for propagation *in vitro* either as explants, *i.e.*, as portions of cellular growth, or as cells in suspension. However, the ease by which cells are handled in suspension makes the cell-suspension method superior for the preparation *en masse* of cultures for viral studies. The procedure employed daily in our laboratory is presented schematically in FIGURE 1.

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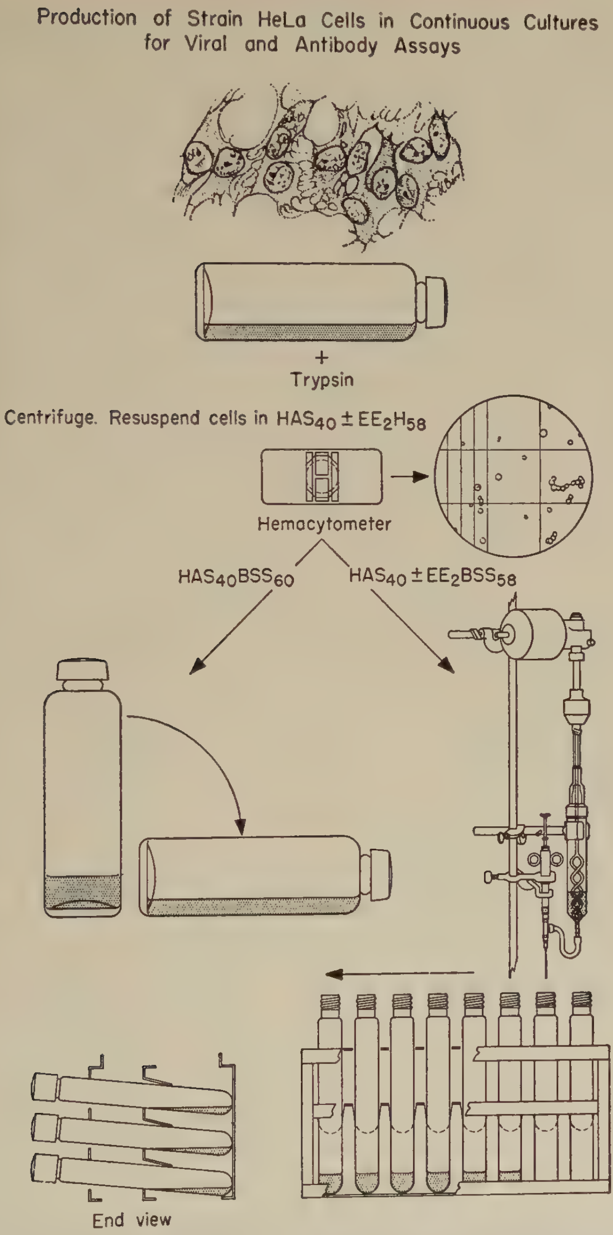
Production of Strain HeLa Cells in Continuous Cultures for Viral and Antibody Assays
+
Trypsin
Centrifuge. Resuspend cells in $HAS_{40} \pm EE_2H_{58}$
Hemacytometer
$HAS_{40}BSS_{60}$
$HAS_{40} \pm EE_2BSS_{58}$
End view

FIGURE 1. Production of Strain HeLa Cells. A schematic representation of the procedure for production of strain HeLa cells for assays in virology. Cells grown in bottles are placed in suspension by the use of trypsin, resuspended in HAS-40, EE-2, H-58, and enumerated in a hemocytometer. After adjustment of the cellular concentration, aliquots of suspension are transferred to bottles and tubes.

Growth of cells. Cells are cultivated *in vitro* on a glass substrate to provide surface for cellular multiplication by the use of a mixture of adult human serum, 40 per cent, and Hanks's salt solution, 60 per cent (HAS-40, H-60). Chicken embryonic extract (EE) may be incorporated in the medium at a 2 per cent level to accelerate cellular multiplication. For mass production, cells in square screw-cap bottles, 200 ml., or for viral assay, cells in test tubes, 16 mm., stoppered by screw caps or by rubber stoppers, are incubated at 36° C. The cultures are placed in a stationary position that permits the fluid to cover fully the cellular growth. Cells in bottles commonly are fed twice weekly. In tube cultures, cells seldom require feeding before use for viral studies. Feeding of cells is accomplished for bottle cultures, either by the addition of 2.5 ml. of 100 per cent adult human serum, HAS-100, to the 8 ml. of medium in each bottle, or by replacement of from half to two thirds of the old medium with new medium, HAS-40, H-60. For tube cultures, the addition of 0.25 ml. of a mixture of adult human serum, 80 per cent, and Hanks's balanced salt solution, 20 per cent (HAS-80, H-20), suffices to feed the cells in each tube. The inoculation of approximately 10^6 cells into a bottle usually results from 10 to 14 days later, in a cellular population adequate for the preparation of subcultures, *i.e.*, 5–10 × 10^6 cells. After transfer, cells are first incubated at 36° C., though commonly when cellular growth covers the glass surface, cultures are placed at 30° C. where cells can be maintained satisfactorily for future use by feeding once or twice weekly.

The above procedure for the growth of strain HeLa cells can be modified. For example, certain human ascitic fluids can be substituted for human serum; the salt solution can be other than that made according to Hanks's formula; glassware of other sizes or shapes is usable; and finally, the temperatures employed for the incubation of cells can vary ± 1° C. without noticeable effects.

Preparation of cells in suspension for transfer. HeLa cells free in suspension for transfer and other purposes are made available by the use of trypsin[10] to release cells from glass and to break up cellular sheets. An inexpensive preparation of commercial trypsin, "Bactotrypsin, 1:250," as a 0.5 per cent suspension in maintenance solution (MS-100), pH 7.4–7.6, exerts satisfactory proteolytic activity without "toxic" effects. The trypsin suspension is twice filtered by employing (a) ash-free filter paper for clarification and (b) a Bush-type filter under positive pressure for sterilization. The filtrate, kept at −20° C., provides a standardized enzyme preparation for periods of several months. Trypsin, in a volume of 8 ml. for cells in a bottle, or 1.0 ml. for cells in a 16-mm. tube, upon incubation at 36° C. for from 60 to 90 minutes and thorough mixing with a serologic pipette, produces a cellular suspension that consists of single cells or groups of no more than several cells. Following horizontal centrifugation of the cellular suspension for 10 minutes at 1000 rpm, the supernatant fluid containing trypsin is discarded and the cells are suspended in a known volume of old, medium, and/or fresh nutritive medium, HAS-40, EE-2, H-58.

The methods that can be employed to make suspensions of strain HeLa cells are, of course, not limited to the procedure that utilizes trypsin. In the past, suspensions of animal cells have been prepared by three general methods: (a) by the use of cells already in suspension, *e.g.*, leucocytes from blood; (b) by the employment of chemical or enzymatic agents for the release of cells from tissue, or from a solid, three-dimensional cultural substrate such as clotted plasma or from solid, two-dimensional substrates such as glass or cellophane; and (c) by mechanical methods, *e.g.*, mincing of tissue or scraping cells from the glass surface of a culture flask. The mechanical method of scraping cells from the glass surface of the culture flask is occasionally used in our laboratory for placing strain HeLa cells in suspension. For routine purposes, however, the trypsin method is used, since it provides suspensions of cells that contain single cells or small clumps of cells, rather than the large clumps of cells that result from scraping a solid sheet of cellular growth.

Enumeration of cells. Cells are enumerated by transfer to a hemocytometer. The suspension of cells should be diluted sufficiently to avoid crowding of cells in the hemocytometer. The total number of cells per cubic centimeter is calculated by employment of the following formula:

$$\frac{\text{Total cells in four chambers (3.6 c. mm.)}}{\text{3.6 c./mm.}} \times 1000 = \text{cells per cc.}$$

Another method for quantitating a cellular population, *e.g.*, the enumeration of cellular nuclei,[11] could doubtless be employed for strain HeLa cells. It is not used by us, however, because it is more time-consuming than is the use of trypsin.

Transfer of cells. The concentration of cells in suspension is adjusted by the addition of fresh HAS-40, EE-2, H-58 to a level that may range from 60,000 to 100,000 cells per cc. for distribution of cells to test tubes, or to bottles. The means employed for dispensing cells to tubes (FIGURE 1) is a modified and simplified design of the apparatus devised by Evans, Earle *et al.*[12] It consists of an all-glass open cylinder with a delivery tube at the base; a helical stirring rod, operated by a 1/18 hp. motor with a rheostatic control, keeps the cells in suspension. A piece of rubber tubing is used to protect the shaft of the stirring rod upon its attachment to the motor. The cylinder is capped by a glass hood. A glass hood attached by a rubber stopper to the dispensing needle helps to prevent contamination. The delivery tube of the cylinder is attached to a Cornwall pipetting unit to enable the rapid delivery of aliquots to test tubes, 16 mm. Commonly, from 25,000 to 75,000 cells are inoculated into each tube. The tubes are stoppered immediately, slanted to permit cells to settle on the glass surface, and incubated at 36° C.

Other types of apparatus have been used to dispense cells in suspension, *e.g.*, ordinary pipettes for small numbers of cultures. A magnetic stirrer can be employed to keep cells in suspension. If it is desirable to have cells over the entire circumference of a tube, cultures of strain HeLa cells may be rotated in a roller drum. For viral assay, it is advantageous not to roll the tubes, so that cells settle in one localized area to facilitate rapid examination microscopically of the cellular growth.

Removal of viral inhibitor(s). Viral inhibitor(s), such as antibody, from the human serum employed for cellular cultivation are assumed to be present in the cultures. Currently, these inhibitors are removed by washing the cells and walls of the culture vessel with balanced salt solution (FIGURE 2) to result in a final dilution of human serum of at least 1:10,000. Washing the cells to remove viral inhibitors by dilution obviously is unnecessary when the medium employed for preparation of tube cultures is known to be free from antiviral substances.

Maintenance of cells for viral cultivation. A mixture of chicken serum, 10 per cent, and maintenance solution, 90 per cent (CHS-10, MS-90), 0.5 ml. is satisfactory for maintaining a normal morphology of from 60,000 to 100,000 cells at 36° C. for from four to seven days. One ml. of this medium usually suffices for about 10 days.

Utilization of Strain HeLa Cells for Assays

Cells of strain HeLa in from one to six days from infection by a virus in the poliomyelitis group,[2,3] or by any of a variety of other viruses,[4-9] undergo rapidly progressive degeneration with the result, over a period of from 12 to 24 hours, of total destruction. Conversely, total inhibition of this fulminant destructive effect of virus results from neutralization of the virus by its homotypic antibody. These facts make easy the interpretation of results of assay, whether for virus or for its antibody. HeLa strain cells can be employed as indicator for the viruses listed in TABLE 1, and probably for other as yet untested viruses.

It should be noted for the viruses listed in TABLE 1 that propagation in cultures of strain HeLa resulted in multiplication for each virus and in destruction of cells by each of the viruses listed with the exception of two: St. Louis encephalitis and Japanese B encephalitis.[7] For these two viruses, cytopathologic effects were observed for from one to several passages. In successive passages, supernatant fluids yielded virus without overt evidence of cellular destruction. Similar findings, suggestive of an equilibrated host-virus relationship, have been reported by others upon the infection of cells in continuous culture by the viruses of lymphogranuloma venereum,[13] Rous sarcoma,[14] and Western equine encephalomyelitis.[15] It is recognized that the absence of a cytopathogenic effect may be limited to the viral strains that were studied and that the capacity to multiply without resultant cell destruction does not necessarily characterize the parent virus.

The methods utilized for the detection of virus, for viral titrations or for antibody assays in cultures of a strain of cells, such as strain HeLa or strain L, are essentially similar to the methods that have been employed with cells cultivated from tissue fragments.

The procedures developed in our laboratory for handling HeLa cells in culture were described in the preceding section of this paper. The procedures for viral detection and titration, and for antibody assay, will next be outlined by employing, for the most part as a model, the strain HeLa cell-poliomyelitis virus system.

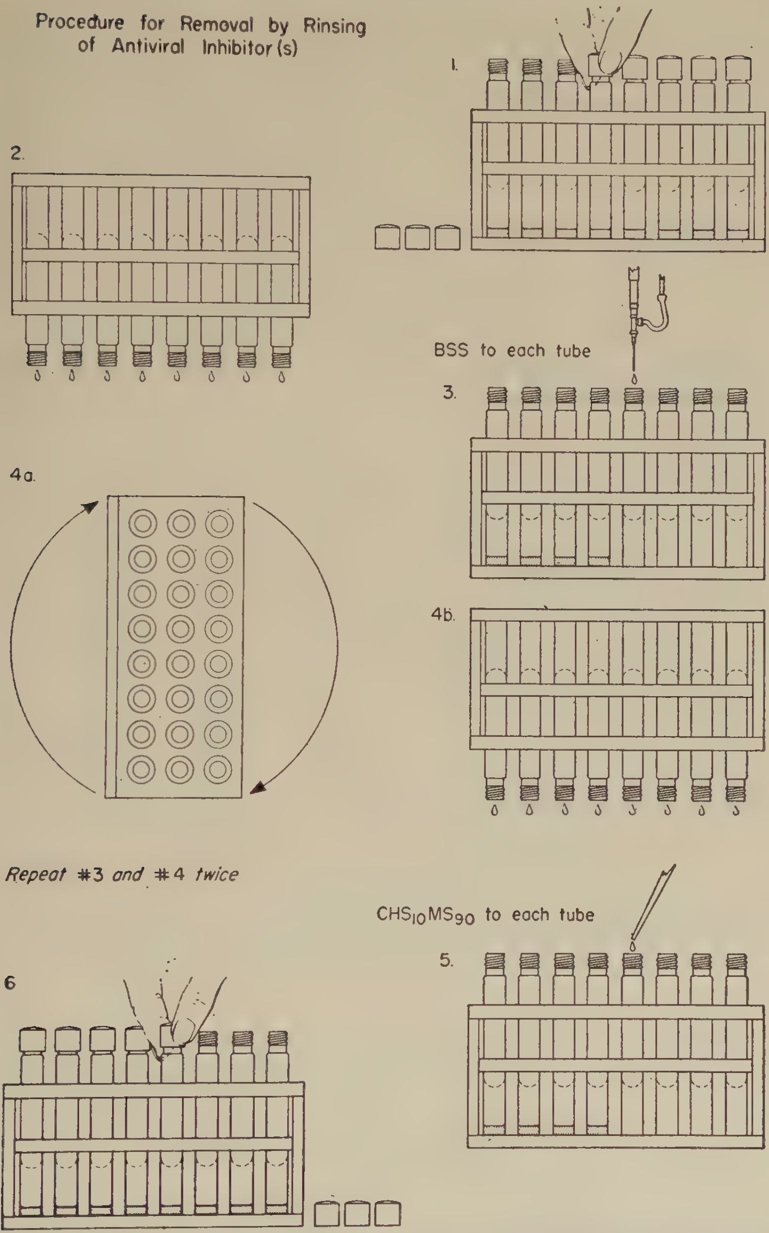
Procedure for Removal by Rinsing of Antiviral Inhibitor(s)
1.
2.
BSS to each tube
3.
4a.
4b.
Repeat #3 and #4 twice
$CHS_{10}MS_{90}$ to each tube
5.
6

FIGURE 2. Procedure for Removal of Antiviral Inhibitor(s). The currently employed method for removal of viral inhibitor(s) from cultures of strain HeLa in preparation for viral cultivation is illustrated: tube cultures are drained of old medium (Steps 1 and 2); cells and the walls of tubes are washed thrice with BSS (Steps 3 and 4); maintenance solution with 10 per cent chicken serum is added to nourish cells during viral cultivation (Steps 5 and 6).

TABLE 1

VIRUSES PROPAGATED IN CULTURES OF HUMAN EPITHELIAL CELLS, STRAIN HELA[1]

Poliomyelitis group[2]
- poliomyelitis, Type 1
- poliomyelitis, Type 2
- poliomyelitis, Type 3

Coxsackie group[4]
- Coxsackie, B-1
- Coxsackie, B-2
- Coxsackie, Texas-14

Arthropodal-borne encephalitogenic group[3]
- Western equine encephalitis
- Eastern equine encephalitis
- West Nile
- St. Louis encephalitis
- Japanese-B encephalitis

Epitheliotrophic group[5]
- vaccinia
- herpes simplex
- pseudorabies

Miscellaneous group[6]
- pseudolymphocytic choriomeningitis

[1] Multiplication and cytologic changes for all viruses except viruses of St. Louis and Japanese-B which were not cytopathogenic.
[2] Reference 2, 3.
[3] Reference 4, 5, 7.
[4] Reference 9.
[5] Reference 4, 5, 6.
[6] Reference 4, 5, 8.

Preparation of contaminated test specimen. The method recommended as routine in preparation for viral assay of any test material of mixed microbial flora for assay of virus, or inhibitor, can be applied to any material that contains a mixed microbial flora (TABLE 2). It depends upon differential centrifugation and antibiotics to control contamination from extraneous bacteria and fungi. This step, of course, is omitted when the test preparation is known to be free from microbial contaminants.

A fecal specimen in a paper carton, as obtained from a hospitalized patient, contains about as widely variable a microbial flora as will obtain for any specimen for virus assay. The treatment, therefore, of such a specimen serves as an example of how grossly contaminated material can be handled. Stepwise, it can be seen from TABLE 2 that an approximate 20 per cent fecal suspension in glass-distilled water containing 2 mgm. per cent phenol red is made up in a 60-ml. heavy duty centrifuge tube. The phenol red, by reflecting extraordinary changes in pH, quickly reveals many of the specimens that prove "toxic" for the cells. The tube is sealed with a pure gum rubber stopper, placed in a horizontal position on a mechanical shaker and subjected for 30

TABLE 2

PREPARATION OF GROSSLY CONTAMINATED SPECIMENS FOR THE ISOLATION OF VIRUS

Success depends on differential centrifugation for control of a mixed microbial population. For example, for feces:

1. Place 6.0 gm. in 60 ml. heavy duty tube (32 x 128 mm.) containing 6–10 beads, 6 mm., 30 ml. sterile glass distilled water, and 2.0 mg. % phenol red.
2. Seal tube and shake horizontally at 450 O.P.M. for 30 min. at 4° C.
3. Centrifuge for 30 min. at 1500 rpm.
4. Discard sediment.
   Centrifuge supernate in Spinco #40 head for 40 min. at 22,500 rpm. (33,000 × G).
5. Add penicillin, 500 units, and streptomycin, 300 μg., contained in 0.05 ml. to clear supernatant fluid.
6. Store at 4° C. for 1 hour and/or at −20 to −70° C. until ready for test.
7. Test for virus by transfer of 0.1 ml. to each of 2 HeLa tube cultures.

TABLE 3

HUMAN EPITHELIAL CELLS, STRAIN HELA (GEY)

Directions for the isolation or detection of virus:

1. 2 tubes human epithelial cells (HeLa), 4–7 days old (80,000–100,000 cells)
2. Remove HuS_{50} EE_2 H_{48}
3. Add 0.9 ml. MS-90, ChS-10
4. Repeat step 3 within the hour.
5. Add inoculum, 0.1 ml.
6. Incubate at 36° C.
7. Examine daily 7 times or until cells are destroyed from the cytopathogenic effect of the unknown virus.

minutes to 450 oscillations per minute. The procedure, thereafter, is carried out as outlined in the protocol. Any comparable angle centrifuge can be substituted for the Spinco. In comment, it is remarkable how effectively the antibiotic mixture controls extraneous microbial contamination when it is added to the final supernatant fluid.

Method for virus isolation and cultivation. The procedure recommended as routine for each test material is outlined in TABLE 3.

Two tubes of HeLa cells with moderately heavy growth on glass (80,000 to 100,000 cells), commonly from four to seven days old, are selected for each test sample and labeled accordingly. Virus inhibitor(s), such as antibody, contained in the nutritive medium is removed as already described. When working with a known virus, the presence of antibody or other inhibitor in the nutritive constituents can be predetermined so as to employ inhibitor-free serum and thereby eliminate steps 2 to 4 in TABLE 3. Cellular cultures for control purposes, of course, must be included.

If a virus be employed with an incubation period requisite for the propagation of virus in excess of five to seven days, the cells in culture should be "fed" with maintenance solution containing 10 per cent chicken serum either by supplementation or replacement of the supernatant fluid.

Cytopathogenic changes. Microscopic examination at magnification times 50 readily reveals evidence for the presence of virus by the rapidly progressive cellular destruction that results from infection. The resultant changes are recorded daily accordingly to an arbitrary standard scale:

0 = no change.

1+ = early degeneration of epithelial cells. Cells may be rounded and transparent. The cells situated peripherally may be swollen. Some cells are abnormally elongate, a few are disintegrated, and a few are partially released from the glass. Similar changes may occur in cultures as a result of nutritional or other disturbance.

2+ = progression of the above changes with evidence of degeneration as shown for cells by distortion, swelling, or shrinkage, and loss of intercellular bridges. The cellular growth shows eversion and partial release from the glass. The nuclei are karyorrhectic or pyknotic.

3+ = cells, for the most part, are totally degenerate with some persisting

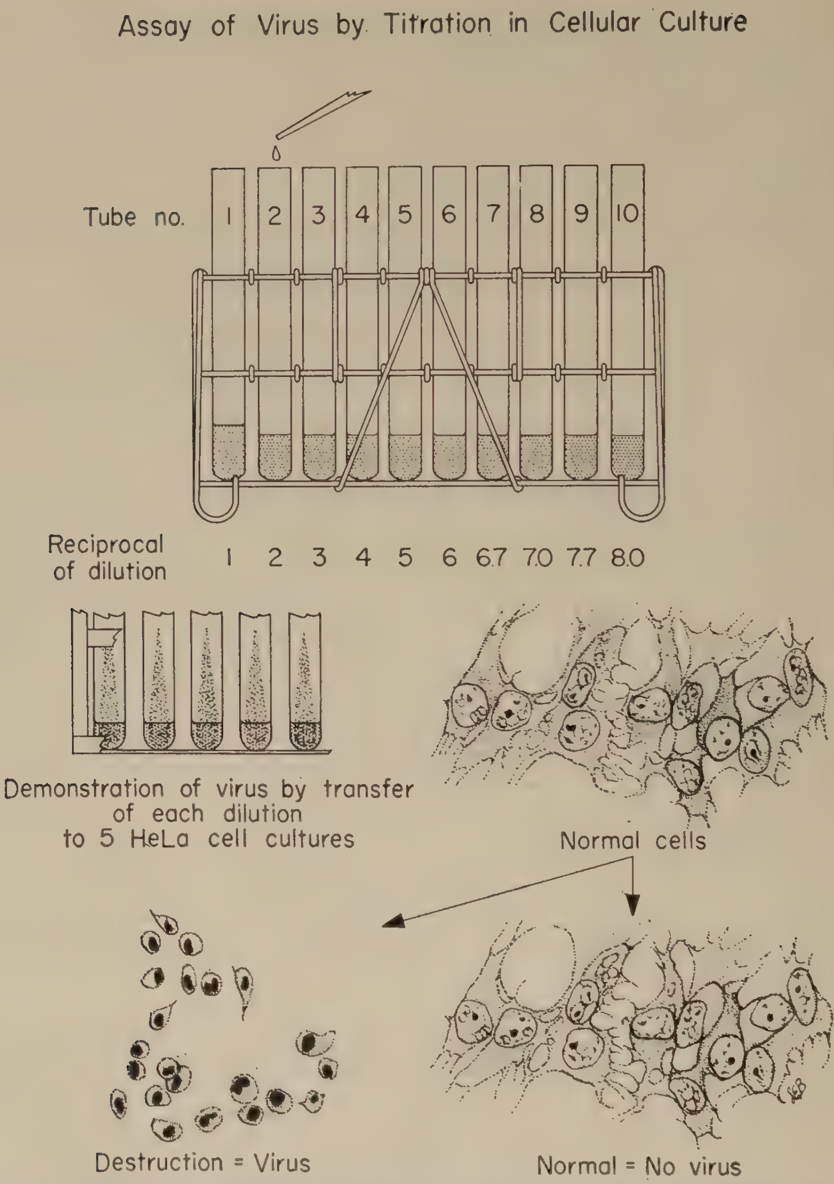
Assay of Virus by Titration in Cellular Culture
Tube no.
1
2
3
4
5
6
7
8
9
10
Reciprocal of dilution
1
2
3
4
5
6
6.7
7.0
7.7
8.0
Demonstration of virus by transfer of each dilution to 5 HeLa cell cultures
Normal cells
Destruction = Virus
Normal = No virus

FIGURE 3. Assay of Virus by Titration in Cellular Culture. Schematic drawing to illustrate the methodology for virus assay and the results of a test. The first set of tubes contains diluent to effect dilutions of the test virus suspension within a known range; the second set of five cellular tube cultures of strain HeLa are for detection of virus; the normal HeLa cells upon infection by virus are destroyed (lower left), or remain unchanged (lower right) in the absence of virus.

as round, granular, distorted cells with pyknotic nuclei and loss of staining affinities.

4+ = dead cells that either slough from the glass wall or remain as shrunken granular cell shadows.

When cellular destruction occurs in the test cultures, commonly in from 12 to 72 hours, it can be assumed that virus in high titer is available in the supernatant fluid for identification by the commonly employed neutralization technic.

Method for assay of virus by titration in cellular cultures. The quantitative assay for content of any test cytopathogenic agent is readily effected by the procedure depicted in FIGURE 3. The test material is diluted within the range desired, commonly in tenfold dilutions from 10^1 to 10^8 (FIGURE 3), by employing as diluent a fluid innocuous for the test agent. An aliquot from each dilution, 0.1 ml., is transferred to HeLa tube cultures in quintuplicate with known amount of MS-90, ChS-10, commonly 0.9 ml., for incubation at 36° C. Daily observation microscopically reveals the end point of infectivity, or titer, by total cellular destruction in the tubes that contain virus, and by cells of normal appearance in tubes without virus. The 50 per cent infective titer ($TCID_{50}$) is calculated by the method of Reed and Muench.[17]

Method for immunologic identification of unknown virus. Known antibody is essential for the specific identification of unknown virus and to establish that any cytologic changes had been produced by virus infection and not from the presence of indeterminant "toxic" material in the test inoculum. The method for production of high titer antiserum as described[3] is readily effected by the use of adjuvants, as recommended by Salk *et al.*[16]

The method employed for the immunologic identification of unknown field strains of cytopathogenic poliomyelitis virus will suffice as an example of the technic that can be employed for any unknown virus. For each test, the protocol outlined in TABLE 4 can be followed. For each test, eight tubes containing from 60,000 to 100,000 HeLa cells (four to seven days old) are employed. Steps 2 to 4 provide for the removal of any antiviral substance in the nutritive fluid, as described above. The eight tubes are labeled for identification of unknown material, or of patient by name and number. Serum

TABLE 4
POLIOMYELITIS VIRUSES

Directions for Typing:
1. Use 8 tubes human epithelial cells (HeLa) 5–10 days old.
2. Replace HuS_{50} EE_2 H_{48} with MS_{100} 0.9 ml.
3. Keep at 36° C. for 1 hour.
4. Replace MS_{100} with MS_{90} MoS_{10}, 0.9 ml., as follows:
    Tubes 1 & 2 MS_{90} + normal MoS_{10}
    3 & 4 MS_{90} + Anti T1 − MoS_{10}
    5 & 6 MS_{90} + Anti T2 − MoS_{10}
    7 & 8 MS_{90} + Anti T3 − MoS_{10}
5. Add inoculum, 0.1 ml.
6. Incubate at 36° C. and examine 7 times daily or until cells are destroyed by polio infection.

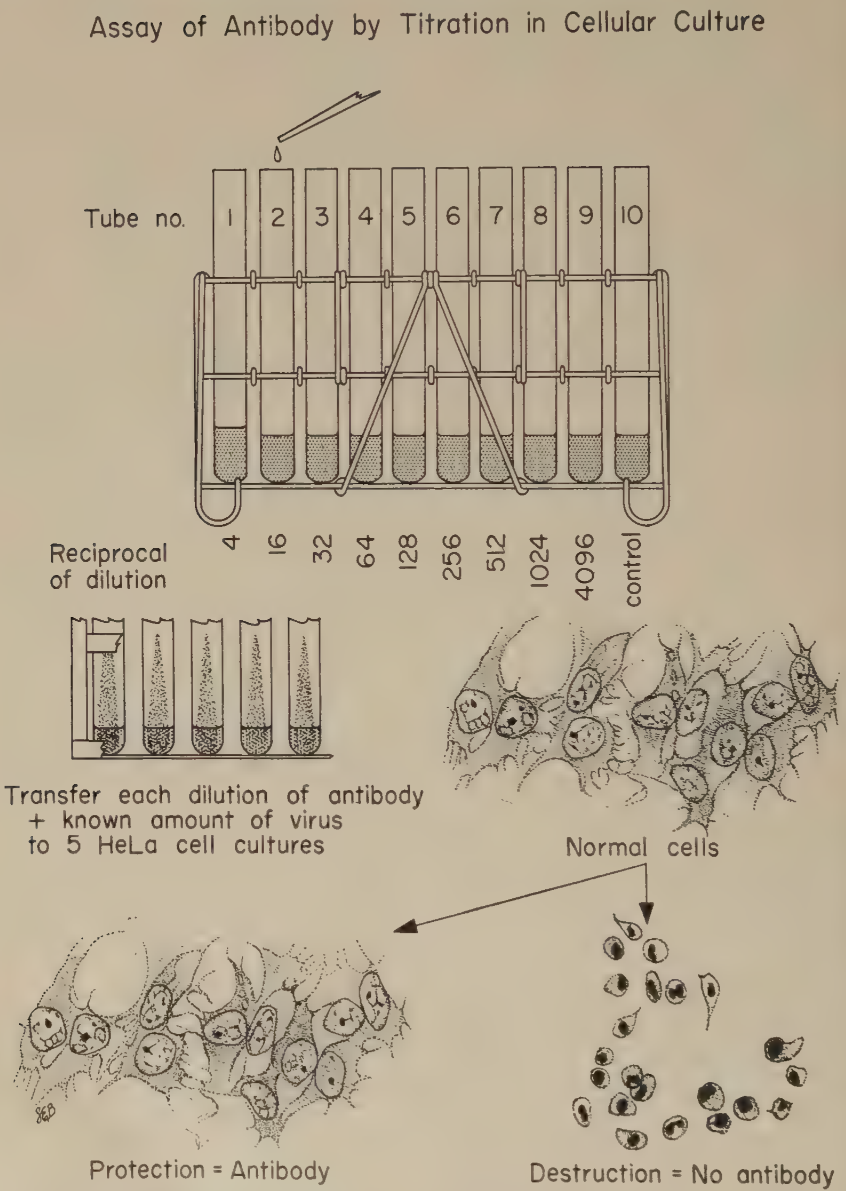
Assay of Antibody by Titration in Cellular Culture
Tube no.
1
2
3
4
5
6
7
8
9
10
Reciprocal of dilution
4
16
32
64
128
256
512
1024
4096
control
Transfer each dilution of antibody + known amount of virus to 5 HeLa cell cultures
Normal cells
SEB
Protection = Antibody
Destruction = No antibody

FIGURE 4. Assay of Antibody by Titration in Cellular Culture. A schematic drawing comparable with FIGURE 3 to illustrate the procedures and results of the technic employed for quantitation of antibody, or other virus inhibitor. The first set of tubes with known amount of diluent permits dilution of the test unknown serum within a known range for mixture with a constant amount of virus in $TCID_{50}$; the second set of tubes illustrates the five cellular cultures employed to test each successive decrement of antiserum for its ability to neutralize the virus in mixture; the drawings of cells depict the change from normal cells at the start to dead cells (lower left) when free virus persists, *i.e.*, unneutralized virus, or to the persistence of unaltered cells (lower left) to illustrate protection from the cytopathogenic effect of free virus.

with known antibody content is added to tubes in duplicate. Normal serum is placed in tubes one and two, antipoliomyelitis Type 1 serum in tubes three and four; Type 2 antiserum in tubes five and six; and Type 3 antiserum in tubes seven and eight. The suspension under test for virus is added, 0.1 ml. to each tube. The tubes are observed microscopically daily. The presence of poliomyelitis virus is revealed in from one to four days, rarely on day five or six, by the destruction of the cells in six of the eight tubes. The unaltered cells in two tubes reflect the protective or neutralizing effect of the homotypic antibody, and thereby reveal the immunologic identity of the strain as Type 1, 2, or 3. This test, as described, constitutes a protection test since antibody is added to the cells in anticipation of the addition of virus subsequently.

Method for assay of antibody by titration in cellular cultures. The quantitative assay for antibody, virus inhibitor, or antimetabolite depends on the ability of the test agent to nullify the cytopathogenic effect of virus for HeLa cells in culture. The principle of the test is essentially similar to other neutralization technics for measuring serum viral antibody except that cellular cultures are employed for detection of residual virus instead of animals or erythrocytes. For example, as presented diagrammatically in FIGURE 4, known decrements of the test material, commonly serum, and a constant amount of virus, commonly from 30 to 100 $TCID_{50}$, are mixed, kept at room temperature for from 30 to 60 minutes and tested for residual infective virus by transfer, 0.1 ml., to each of five HeLa cell cultures for incubation at 36° C. Daily observation microscopically establishes the end point, as revealed by the tubes with the least amount of antibody and unaltered HeLa cells, *i.e.*, complete protection by antibody of the cytopathogenic effect of the fixed amount of virus in each serum-virus mixture.

The principal use of strain HeLa cells for viral titrations, or antibody assays has been to date for studies with poliomyelitis virus. Other viruses destructive for strain HeLa cells, however, were studied recently (TABLE 1).[4-9] Strain HeLa cellular cultures, as described for the virus of poliomyelitis, have been employed for titrations that were concerned with the viruses of herpes simplex, pseudorabies, vaccinia, Western equine encephalomyelitis, Eastern equine encephalomyelitis, West Nile, pseudolymphocytic choriomeningitis, and three immunologic types in the Coxsackie group.

From the results of these and other studies, it is evident that the practical application of cellular cultures for virus detection and titration, and for antibody assay depends upon (a) the existence of a host cell-virus relationship that results in destruction of the cell, and (b) the ready availability of cellular cultures in quantity.

Discussion

Stable strains of mammalian cells in continuous culture simplify the study of viruses and of viral infections. It has been demonstrated in our laboratory, for example, that a stable strain of human epithelial cancer cell (strain HeLa, Gey) in continuous culture possesses distinctive inherent qualities which make these cells excel in usefulness. A salient quality is the ease of adaptability of

HeLa cells to large scale cultivation. To cite other examples from studies reported or underway: (a) this strain of human cells supports the growth of poliomyelitis virus and a variety of other viruses; (b) the capacity for growth in fluid media on glass in the absence of plasma has simplified cultivation; (c) these cells are readily released by trypsin to provide single cells free in suspension; (d) HeLa cells in suspension can be quickly enumerated by counts in a hemocytometer and readily dispensed by a simple apparatus in aliquots to provide large numbers of replicate cultures or to provide single cells for intimate host cell virus studies; (e) the cells settle and stick to glass walls kept stationary at 36° C. in a nearly horizontal position to reproduce fivefold to tenfold in 5 to 10 days; (f) the stability of these cells while in continuous serial passage makes it apparent that the strain can be maintained in perpetuity by continuous transfer in series; (g) under the extraordinary environmental conditions that result from shipment by train and plane, these cells possess hardiness and a capacity for renewed proliferation upon adding nutrient media.

The usefulness of stable strains of normal and malignant cells in continuous culture for the study of viruses has been previously reported in several papers.[2-9, 13-15, 18-22] For example, Gey and Bang, in 1939,[13] recorded the successful propagation of the virus of lymphogranuloma venereum in continuous roller tube cultures of thyroid fibroblasts for more than seven months. The virus in culture fluids was demonstrated by its lethal effect for mice, its specific neutralization by known antibody, the production of a positive Frei test in patients, and the demonstration of characteristic intracellular changes in the fibroblastic cells. The lethal effect for mice was lost after 111 days in continuous culture, but the culture fluid thereafter retained the ability to elicit cytologic change and a positive Frei test. Nine years later, Sanford, Earle, and Likely[23] reported the derivation of a pure strain of mammalian cells by single cell isolation (strain L, Earle). The single cell of origin was isolated from a strain of normal mouse mesenchyme cells that had been treated with methylcholanthrene and had subsequently become malignant. The ability of the pure strain cells to produce tumors upon animal inoculation apparently had been lost before the single cell strain was isolated.[24] The progeny, in successive passages, retain the characteristically altered morphology and the ability to produce colonies from scattered isolated cells on glass. The L strain is unique in being the first pure strain derived from the single mammalian cell so far reported. This singular accomplishment by Sanford, Earle, and associates has been extended by the development, for L strain cells, of methods for making replicate culture *en masse*.[12]

L strain cells are ideally qualified for the study of mammalian virus-cell relationships since cell clones of this pure strain and a known virus provide a system which closely resembles the bacteriophage-bacterial system. Indeed, it was the genetic homogeneity of these cells which led to their use in this laboratory. To date, it has been reported that the L strain of mouse fibroblastic cells supports the propagation *in vitro* of the viruses of pseudorabies,[25] herpes simplex,[25] lymphocytic choriomeningitis,[26] pseudolymphocytic choriomeningitis,[26] encephalomyocarditis, MM strain,[26] and Western equine encephalomyelitis virus.[15] Contrariwise, this cell strain did not support under similar laboratory conditions the growth of poliomyelitis virus,[27] the GD VII strain of mouse encephalomyelitis virus,[27] or Rous sarcoma virus.[14] Infection of L strain cells by the viruses of herpes and pseudorabies resulted in intranuclear inclusion bodies of Type A.[25] The consequence of infection by the viruses of pseudorabies, herpes simplex, and pseudolymphocytic choriomeningitis viruses was cellular destruction. Lymphocytic choriomeningitis produced no cellular alteration. The production of pseudorabies and herpes simplex virus in cultures of these cells was related to the metabolic activity of the cells. For example, the production of virus was greatest when the cells were maintained in the physiologically active state by the use of horse serum-embryonic extract medium at 37° C. Less virus resulted at a lower level of metabolic activity provoked by the use of diluted ox serum ultrafiltrate at 37° C. Viral production was minimal, or not detectable, in cellular cultures without apparent metabolic or reproductive activity consequent to the utilization of the maintenance solution at 22 to 25° C. Infection of L strain cells by vaccinia virus resulted in agglutination.[28] The specificity of this response was established by the demonstration that vaccinia antibodies inhibited agglutination. The viruses of mumps and influenza A under the experimental conditions employed failed to agglutinate L strain cells. Chambers and Evans[15] kept Western equine encephalomyelitis virus in cultures of L strain cells with an initial cytopathogenic effect. This effect was soon lost despite evidence that viral propagation persisted for eight months. Similarly, we[7] found for HeLa strain cells that the viruses of St. Louis encephalitis and Japanese B encephalitis propagated after from one to several passages without overt evidence of cytologic changes.

Other opportunities for studies of intimate host cell-virus relationships by the utilization of stable cell strains in continuous culture were discussed previously.[29]

Summary

The studies that concern the application of mammalian cells in continuous culture for assays in virology are reviewed. Studies from our laboratory have provided evidence of the ready applicability for assay purposes of a stable strain of human epithelial cancer cell, strain HeLa, Gey. Strain HeLa cells may be employed readily, inexpensively, and effectively for: (1) the production of virus, commonly from 10^5 to 10^7, $TCID_{50}$, in one to four days; (2) the isolation of virus from patients, animals, arthropods; (3) the detection and quantitation of virus; (4) the detection and quantitation of specific antibody by employing the tissue culture neutralization technic; (5) the specific identification of field strains of virus by employing known type antisera; (6) the screening of test agents for chemotherapeutic or antibiotic effect; (7) comparative studies of the metabolic activity of virus infected and noninfected cells; (8) the mass production of cancer cells for biochemical studies; and (9) the assay of viruses, specific antibodies, antimetabolites, antibiotics, chemotherapeutic agents, and other materials.

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THE APPLICATION OF TISSUE CULTURES TO THE PREPARATION AND ASSAY OF VIRAL ANTIBODIES*

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Historical Introduction

Almost from the time of their introduction as tools of research, tissue-culture techniques have been employed on occasion by virologists. Until comparatively recently, however, this group of investigators has applied these methods largely to the continued propagation of viruses *in vitro.* As a result, it has been possible for some time to compile imposing lists of those agents that have been propagated for varying periods of time in tissue cultures.[1, 2] Because of this preoccupation with the mere growth of virus, which, it must be admitted, was largely enforced by the limitations of knowledge and techniques then available, one encounters relatively few investigations in the earlier literature that attempt to elucidate, in the simplified system provided by the tissue culture, such fundamental problems as the mode of attachment of the virus particle to the cell; the manner in which it enters the cell; the mechanism of its intracellular multiplication; or the factors whereby the cell is rendered resistant to attack by the virus. Likewise, with a few notable exceptions, the potentialities of tissue cultures lay neglected as practical and convenient means of assaying viral infectivity, of measuring neutralizing antibodies, of altering viral pathogenicity, of producing large quantities of virus suitable for study of its physical and chemical properties, or for use as an immunizing agent. Failure to explore more vigorously such possible applications of the method may be attributed chiefly to the fact that no simple and rapid way had been found to determine within the culture itself whether or not multiplication of virus had occurred. To demonstrate the presence of virus and evaluate its activity, it was usually necessary to inoculate the materials of the culture into appropriate experimental animals. From the practical standpoint, therefore, cultivation of a virus *in vitro* seemed to offer little advantage over that in the living animal. Furthermore, before the advent of antibiotics, the elaborate precautions essential to prevent bacterial contamination presented an impediment to the preparation and use of tissue cultures on a scale required for the investigation of various phases of viral behavior. While such considerations discouraged many from invoking the technique as a fruitful method for the study of these agents, a few investigators in the past, recognizing its inherent advantages, established a number of significant and useful facts. I shall recall certain of the more remarkable of these earlier studies, since they are relevant to much of the more recent experimental material I shall subsequently mention. Carrel,[3] in 1924, clearly saw and then proceeded to demonstrate the value of tissue culture as applied to the problem of the pathogenicity of viruses. Employing pure cultures of chicken macrophages and fibroblasts, he obtained evidence that the

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macrophage alone appeared capable of supporting the growth of Rous sarcoma virus and that, under its influence, it often assumed at least one of the characteristics of malignancy: *i.e.*, increased capacity to digest the medium. His observations also strongly suggested that multiplication of this virus within the cell does not lead to its destruction. On the contrary, cell division may proceed unimpeded and be accompanied by continual production of virus over long periods. In these experiments of Carrel, four important facts or concepts were given emphasis for the first time: (1) cells in culture infected with a virus may show alterations in their behavior that can be directly observed; (2) large quantities of virus may be generated in the culture, and this generation may continue indefinitely; (3) certain viruses may establish with the cell a relationship that may be regarded as essentially symbiotic; (4) one type of cell may be susceptible to viral infection and another resistant.

The abundant and persistent production of Rous sarcoma virus in plasma clot cultures led Carrel, working in collaboration with Rivers,[4] to a study of vaccinia virus under these conditions, and it was found that this agent behaved in a similar manner. This observation, in turn, induced Rivers and his associates[5, 6, 7] to explore the possibilities of the suspended-cell culture which had then recently been developed for the production of virus to be used for the vaccination of man instead of calf lymph. It was demonstrated that, for this purpose, sufficient virus free of bacteria could be readily produced in cultures of chick-embryo tissues. Unfortunately, the immunizing properties of the virus became reduced after repeated passage *in vitro*, and this tissue-culture vaccine was never adopted for use as routine.

Rivers's investigations extended over the earlier years of the thirties. During this same period, the classical experiments on the cultivation of yellow-fever virus in tissue cultures were carried out by Theiler and his associates[8, 9] resulting in the production of the attenuated strain D17 that is employed in vaccination against this disease. Although this strain is cultivated today in the chick embryo, infected tissue-culture material was used for a time as vaccine. The history of the yellow-fever vaccine provides an example that cannot be too often recalled, of how the tissue culture can be effectively applied, not only in the development of a virus of attenuated virulence suitable for immunization, but also in the routine production of a vaccine. The recollection of this distinguished chapter in the history of virology is, I think, particularly relevant at this time, when efforts are being made in several directions to produce vaccines suitable for use against poliomyelitis.

The importance of being able to recognize the presence of virus in cultures by direct inspection was, as I have indicated, fully appreciated by Carrel. Other workers had also been aware of its significance. For example, Steinhardt and her collaborators,[10] were in part stimulated, in 1913, to undertake the cultivation of vaccinia virus in tissue explants by the hope of demonstrating the production of inclusion bodies *in vitro*. They failed, however, to find Guanieri bodies in their cultures, although they succeeded in demonstrating multiplication of the virus. Other earlier workers[11, 12] likewise were unable to observe the typical inclusions of vaccinia in infected tissue cultures. Indeed, it was not until 1929 that unequivocal evidence for the appearance *in vitro* of such morphologic indicators of viral activity was obtained in this country by Rivers, Haagen, and Muckenfuss[13] with the viruses of vaccinia and herpes simplex and, in England, by Andrewes,[14] with Virus III. Confirmation of these findings was soon forthcoming from other laboratories and, on the basis of these phenomena, experiments were undertaken, notably by Rivers and Andrewes,[15, 16] which led to a clearer understanding of the mechanism of immunity to viral agents. During the course of these studies, Andrewes[16] showed that it was possible to titrate viral neutralizing antibody in tissue cultures taking the suppression of inclusion-body formation as an index of neutralization. I have mentioned these well-known studies because they represent the first analyses of the effect of viral antibodies in infected tissue cultures as well as the first use of cultures for the assay of these antibodies.

Although the presence of inclusion bodies in tissue cultures may be regarded as presumptive evidence for viral proliferation, their development may be somewhat irregular and the procedures involved in rendering them visible are obviously time-consuming and laborious, since carefully fixed and stained preparations must be examined. Moreover, the presence of these structures cannot be taken as conclusive evidence of viral activity, since various investigators have occasionally observed similar formations in the cells of tissue cultures to which no virus had been added. For these reasons, and also because many viruses do not produce inclusions, assay of viral infectivity and viral neutralizing antibodies based on the occurrence of inclusion bodies proved impractical as routine procedures.

Other manifestations of the effect of certain viruses on cultured cells had also been observed from time to time by various workers. Their value, however, as indices of infection was not at first completely perceived. Plotz and Ephrussi,[17] in 1933, found that cells infected with fowl-plague virus failed to migrate or grow normally in plasma clot cultures, and Ivanovics and Hyde[18] noted degenerative changes in cultures of various tissues infected with Virus III. In unpublished experiments with Relova, in 1940, we observed in roller-tube cultures rapid and usually complete destruction of chick embryonic cells infected with equine encephalomyelitis virus. But to Huang[19] must be given the credit of first utilizing the destructive capacity of equine encephalomyelitis virus as a means of measuring infectivity and assaying the neutralizing capacity of immune sera. Huang first showed that the infectivity of this agent could be accurately assayed *in vitro* by determining the highest dilution that prevented migration of chick embryonic cells in plasma cultures. Then, using mixtures consisting of a constant amount of specific antiserum and varying quantities of virus, he was able to measure the capacity of the serum to prevent cell injury by the virus. Comparative experiments in tissue culture and in mice revealed close agreement both in measurements of infectivity and in neutralizing antibody. Indeed, the tissue-culture method appeared under certain circumstances to afford a more sensitive means of assaying these properties. Here then, at last, was revealed a simple and apparently accurate method for the quantitative study *in vitro* of those viruses that possess the capacity to injure and kill the cells that support their growth.

In 1948 and 1949, Weller, Robbins, and I[20, 21] found that the viruses of poliomyelitis could be grown in various human extraneural tissues in suspended-cell cultures. Histologic examination of the tissue fragments from such cultures revealed injury and death of cells. These changes contrasted strongly with the condition of the cells in materials from uninoculated control cultures. It was soon noted that the cytopathogenic effect, as we came to call it, of these viruses was more clearly, rapidly, and easily revealed in roller-tube cultures.[22] In this medium, the infectivity of suspensions of virus could be measured within a few days by noting the highest dilution initiating death and disintegration of the cells. It was also shown that the cytopathic changes induced by the virus were prevented by type specific immune serum which obviously provided a convenient method whereby the latter could be assayed for neutralizing antibody. Others[23, 24, 25, 26, 27] soon found that poliomyelitis viruses might also be propagated in monkey tissues and that they exerted comparable cytopathogenic effects in these materials.

At about this time, another manifestation of viral multiplication in tissue culture that could be readily recognized was described by Weller and Enders,[28] who noted the regular emergence of viral hemagglutinin in suspended-cell cultures of mumps and influenza viruses. Simply by adding a portion of the culture medium to red cells after an appropriate interval, following inoculation of the virus, it could be determined whether or not growth of the agent had occurred. This procedure is valuable for the study of viruses such as mumps and influenza that do not regularly exert clear-cut cytopathic changes. It has already been employed in the evaluation of infectivity and neutralizing antibodies by Fulton, in England,[30] and by Kunz[31] and Gajdusek,[32] in our laboratory.

From this imperfect sketch of efforts to fashion the tissue culture into a truly effective device for the production and assay of viruses and their antibodies, it is apparent that, only recently, has this goal been attained. Within the past few years, however, enough has been learned to suggest that the method will take, among procedures available to the virologist, a permanent place alongside the experimental animal or that, in certain situations, it may even supplant the animal. Accordingly, in the remainder of this paper, I shall briefly review and discuss certain of these recent advances in so far as they relate to the production of viral antigens and the detection and titration of viral antibodies.

The Production of Viral Antigens

Quantities of virus produced. The discovery made by workers in the past, that large quantities of virus may be elaborated in tissue cultures, has been satisfactorily confirmed by modern investigations. As measured by infectivity, for example, titer representative strains of the three types of poliomyelitis virus have been shown to attain levels in the fluid phase of cultures of extraneural tissues equivalent to, or in excess of, those reached in the infected nervous system of monkeys or mice. In TABLE 1 are presented a few data obtained by different workers illustrative of the titers that may be expected when these agents are propagated in the usual types of cultures now being employed. In the case of certain other viruses that have been recently investigated, such as influenza[30] or mumps,[31] the quantities of virus produced have been less than

TABLE 1

REPRESENTATIVE TITERS OF POLIOMYELITIS VIRUSES GROWN IN TISSUE CULTURE BY VARIOUS INVESTIGATORS

Investigator	Strain of virus	Tissue culture titer*
Robbins *et al*[33]	Brun. (I)	5.8†
	Lans. (II)	5.5†
	Leon (III)	5.5†
Younger *et al*[26]	Mah. (I)	4.0‡
	Sauk. (III)	4.5‡
Ledinko *et al*[27]	WS (I)	4.0‡
	Y-SK (II)	3.0‡

* Expressed as log ID_{50} per ml. of culture fluid.
† Virus grown and titrated in roller cultures of human embryonic skin and muscle.
‡ Virus grown and titrated in roller cultures of monkey testis.

those developing in embryonated eggs. However, not inconsiderable amounts appeared in the culture fluids. A distinct advantage of the culture, as compared with the living animal for the manufacture of virus, lies in the fact that, with certain agents, the virus continues to multiply and to appear in the fluid phase over prolonged periods. By changing the medium at frequent intervals, accordingly, large quantities of infectious material may be accumulated from a single culture. In either roller tubes or suspended-cell cultures of poliomyelitis virus, the level of virus production may remain at a significant level over a period of many days.[33, 34] As an illustration of this fact, the quantities of the Lansing strain, appearing every two days in the fluid of two sizes of roller cultures of human embryonic tissues are shown in FIGURE 1. The culture method for the production of virus to be used as antigen affords yet another advantage over the intact animal, since it provides a means of reducing the amount of tissue derivatives and other foreign proteins to a minimum. I shall indicate later, more concretely, how this may be accomplished when I allude to the preparation of complement-fixing antigens from tissue-culture materials.

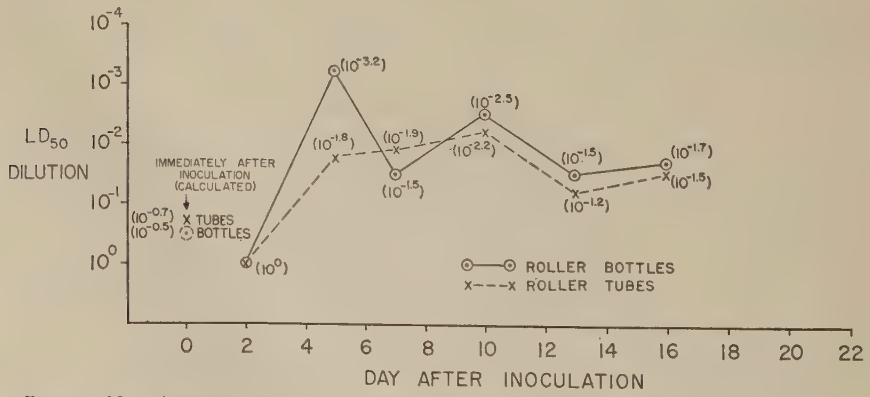

FIGURE 1. Mouse infectivity titers (LD_{50}/ .03 ml.) of pools of fluids removed from roller bottle and tube cultures of mixed human embryonic tissues inoculated with Lansing tissue-culture virus (reproduced by permission of the *Journal of Immunology*).

TABLE 2

TITERS OF POLIOMYELITIS VIRUSES GROWN IN HUMAN EMBRYONIC SKIN-MUSCLE TISSUE IN TWO TYPES OF CULTURE

Virus	Culture for growth	Titers‡ obtained in RT cultures	Av. of titers
Brun (I)	RT*	5.8 5.2 5.5 5.8 5.8 5.5 5.2 4.8 3.8 5.2	5.3
	SCC†	5.5 3.8 5.2	4.8
Lans (II)	RT	5.5 5.5 5.5 5.5	5.5
	SCC	4.5 4.5 3.7 4.5 3.8	4.2

* Roller tube culture.
† Suspended cell culture.
‡ Expressed as neg. log ID_{50}/ml.

We may conclude, then, from this short review of a small portion of data derived from modern experiments with viruses in tissue culture, that Carrel correctly saw in this method a means of producing large quantities of infected materials easily, cheaply, and in a relatively pure state.

Conditions Influencing the Yield of Virus

It has long been recognized that the conditions of cultivation markedly influence the yield of virus in tissue culture.[1, 2] Numerous illustrative data might be cited, but here I shall limit myself to what lately has been learned concerning certain of the factors that affect the multiplication of the poliomyelitis viruses.

(1) *Type of culture.* The type of culture selected for cultivation may be of significance, although we do not yet have sufficient comparative data to warrant any final conclusion in regard to the superiority of one over another. It is evident, however, from results such as are summarized in TABLE 2, that the yield of virus in certain instances may be increased considerably, depending on the type of culture selected. In this table are recorded infectivity titers obtained in various experiments, in which the Brunhilde and Lansing strains were propagated in roller tubes and in suspended-cell cultures. In both, the tissue consisted of fragments of human embryonic skin and muscle. The assays of the fluids removed from these cultures were all carried out in roller tubes.

Although the number of determinations on materials from the suspended-cell cultures is small, it is apparent that, with the Brunhilde strain, as much virus may develop in this type of culture as in the roller tube. In contrast, the quantity of Lansing virus appearing in the fluid of suspended-cell cultures was only about one tenth of that found in the roller-tube cultures.

(2) *Types of tissue.* It would seem, *a priori*, that the types of tissue or cell might represent a controlling factor in the yield of virus. There is, however, relatively little information available which clearly illustrates such differences, except where the tissues of one species may support a viral multiplication which those of another fail to do. In the case of the poliomyelitis viruses, certain differences in their capacity to grow in various susceptible tissues have been observed, but additional comparative experiments are to be desired. Thus

TABLE 3

INFECTIVITY TITERS OF POLIOMYELITIS VIRUSES GROWN IN ROLLER TUBE CULTURES OF MATURE HUMAN TISSUES

Virus	Tissue for growth	Tissue for titration	Titer*
Brun (I)	uterus	emb. sk-m	5.8
	uterus	uterus	5.5
	prepuce	uterus	5.2
Lans (II)	uterus	emb. sk-m	3.8
	uterus	uterus	4.3
	prepuce	uterus	4.5
Leon (III)	uterus	emb. sk-m	6.2
	uterus	uterus	4.6
	prepuce	uterus	5.5

* Neg. log ID_{50} per ml.

Weller, Enders, and Robbins[34] measured the infectivity of the fluid phase from suspended-cell cultures of nine different human embryonic tissues. The highest titers, which did not differ significantly among themselves, were obtained in intestine, kidney, and skin and muscle tissues; the lowest in spleen, lung, and liver. Intermediate quantities of virus were found in cultures of adrenal brain and heart tissues. Although it is probable that these differences, in most cases, depend on the nature of the tissue, other factors may have occasionally been responsible. For instance, the cultures of liver failed to exhibit much evidence of viability after a few days.

With the possible exception of human testicular tissue[33] obtained from elderly men, we have not observed striking disparities between the amounts of virus produced by other mature tissues, such as those of the uterus or prepuce. Titers indicating the yield of the three prototype viruses that may be obtained in roller-tube cultures of uterine and prepucial tissues removed at operation are presented in TABLE 3. The data suggest that possibly the Lansing virus does not attain as high a level in these tissues as in embryonic skin and muscle.

High yields of virus have been obtained by Scherer, Syverton, and Gey[35] in cultures of the HeLa strain of human carcinoma cells. Infectivity titers, as determined in HeLa cell cultures, have ranged between 5 and 7 (neg. log ID_{50}/ml.).

In monkey testis, the quantities of virus elaborated in roller cultures usually appear to be somewhat lower than those produced by certain human tissues, as suggested by the data I have shown in the first slide. Inspection of the protocols published by Melnick and his associates, and by Salk and his co-workers, will confirm this impression.[26, 27] More recent experiments made by Salk, however, have made it clear that considerably more virus develops in roller-tube cultures of monkey kidney.[36] For this reason, Salk selected the kidney for use in the preparation of vaccines. In this connection, it is of interest to note that Frenkel[37] has employed lingual mucosa as the most efficient bovine tissue for the production of foot and mouth disease virus.

(3) *Size of inoculum.* When grown in certain types of culture, it has been found that the yield of virus is markedly affected by the size of inoculum. A

recent example of this behavior is recorded by Womack and Kass[38] who found that influenza A virus in allantoic sac cultures failed to attain maximal growth when less than 0.5 hemagglutinating unit was inoculated. Similarly, in suspended-cell cultures of human embryonic skin and muscle, we observed that small inocula of Lansing virus resulted in low virus production.[34] Multiplication of this agent, however, is quite different in roller-tube cultures. In this system, irrespective of the type of tissue, maximal yields of virus have been regularly observed following the addition of inocula ranging from 10,000 to 10 ID_{50}.[33] It is probable that this striking difference in capacity of small quantities of virus to multiply in these two types of culture is to be attributed to the superior conditions offered by the roller-tube method for the survival and multiplication of the cells. Syverton and his associates[35] have likewise shown in cultures of HeLa cells that the final yield of virus is quite independent of the concentration of virus inoculated.

(4) *Other factors.* In the past, it has been asserted that a variety of factors other than those I have just mentioned, affect the multiplication of viruses, including such factors as the quantity of tissue, the composition of the medium, the ratio of fluid to tissue, the ratio of fluid volume to the size of the vessel, and agitation or rotation of the culture. In the case of the poliomyelitis viruses, these factors have not yet been thoroughly investigated. From what has been already noted, however, it would seem unlikely that they will prove to be of major importance.

Within comparatively wide limits, accordingly, the quantity of tissue can be varied without apparent change in production of virus.[27] So far, Syverton and his co-workers,[35] have given us the most satisfactory demonstration of this fact in showing that the amount of virus developing was the same in two sets of cultures in which the number of cells varied by a factor of 10.

It is also clear that the size of the vessel, and consequently the relation of fluid to air space, plays no significant role, since various investigators have employed vessels of widely differing capacities. For the production of large quantities of virus we have used bottles of 250 ml. capacity with 30 ml. of medium and large amounts of tissue.[39] These bottles have been rotated slowly under the same conditions as roller tubes. The yields have been of the same order of magnitude as those encountered in the smaller vessel. Miller[40] obtained titers equivalent to those found in roller tubes with all three types of poliomyelitis virus cultivated in Roux bottles of 1-liter capacity. He used coagulated plasma as a base for cell growth. In certain experiments he also successfully employed six-liter rectangular bottles.

Continuous movement of the culture also seems unnecessary, since it has been demonstrated by Melnick and Riordan[41] and Scherer and Syverton[35] that, in stationary cultures, the yields of virus may be as high as those obtained in roller tubes.

From this fragmentary survey of certain factors that may affect the yield of a virus in tissue cultures it is difficult to draw any general conclusions. However, we may affirm that maximal growth occurs regularly in systems where the metabolism of the cells is well maintained, and where the greatest opportunity for contact between the cell population and the virus is afforded. If these conditions are provided, variation in certain others is apparently of little importance.

Concentration of tissue-culture virus in the preparation of antigens. The concentration of viral antigens present in tissue-culture fluids can be easily accomplished because it has been found possible to reduce the quantities of extraneous substances of high molecular weight to a very low level. This result may be effected in the case of plasma type cultures either by the initial use of a relatively simple medium such as the lactalbumin digest medium of Melnick[41] or by substitution of such a medium for one containing embryonic extract and serum at the time the virus is inoculated. These procedures have been found not to decrease the yield of poliomyelitis virus. Infected fluids prepared by the method of substitution have been concentrated in our laboratory 300 to 600 times by dialysis under negative pressure through collodion-impregnated alundum shells. Antigens consisting of each of the three types of poliomyelitis viruses prepared in this manner have been employed successfully by Svedmyr and his associates[39, 42] in complement fixation tests with human and monkey immune sera. The capacity of such concentrated antigens after inactivation to stimulate antibodies in experimental animals has not yet been investigated. *A priori*, however, it would seem likely that they might prove more efficient in this respect than unconcentrated infected tissue-culture fluids.

Tissue-culture virus as vaccine for use in man. In spite of the inherent advantages of the tissue culture for the production of viral vaccines for human use, none, as I have already indicated, has yet been adopted as routine for this purpose. Lately, however, it has been proposed to employ the method in the manufacture of a vaccine against poliomyelitis, for it had previously been demonstrated that virus appearing in tissue-culture fluids was capable of stimulating antibody production in the animal body. Milzer and his associates[43] and Enders and his co-workers[44] had observed that, following intramuscular or intraperitoneal inoculation of active Lansing tissue-culture virus, mice might be protected against the lethal effect of an intracerebral injection of the homologous strain. Salk and his co-workers,[45] a little later, described the development of virus-neutralizing antibodies in monkeys following the injection of the three types of virus propagated in cultures of monkey tissues and then inactivated with formalin. On the basis of these results, attained with inactivated viruses, experiments were carried out by Salk in man.[46] Again, type-specific antibody formation was demonstrated in the majority of subjects following administration of the vaccine. In persons presenting evidence of previous infection, as indicated by the presence of low antibody levels before vaccination, the antibody response was prompt and unequivocal. In contrast, individuals in whom no neutralizing antibody could be demonstrated prior to vaccination usually responded with low levels, or they failed to develop demonstrable antibody as revealed by neutralization tests performed in tissue cultures. Nevertheless, since there are many indications that even very low concentrations of circulating antibody may protect against infection when it takes place by an extraneural route, these preliminary results have led to plans for additional experiments in man that are to be undertaken in the near future on a large scale.

Assay of antibodies in tissue culture. I shall now comment on the application of tissue-culture methods to the assay of virus-neutralizing antibodies. Again, the data that I shall mention have been largely derived from studies on the poliomyelitis viruses. This choice of material seems to me appropriate since assay of antibody in tissue culture is being employed at present in the diagnosis of doubtful cases of poliomyelitis,[42] in the revelation of past inapparent infections,[47] and, as I have already pointed out, in the evaluation of specific prophylactic agents. As yet, however, no standard procedure for the performance of neutralization tests has been adopted. Realization of the great benefits that would follow if a standard procedure could be devised and generally accepted by all workers in the field has also prompted me to make this choice.

General techniques employed in neutralization tests. Two general techniques are available for the performance of tests for virus neutralizing antibodies. In the one, the concentration of the serum is held constant, while the concentration of virus suspension is varied. In the other, the concentration of virus is fixed, while that of the serum is varied. Analyses of the reaction can be made by carrying out "box" titrations in which varying concentrations of both reagents are employed, but this type of experiment is too involved to be suitable for routine tests. Of late, the majority of workers who have used the tissue culture in neutralization tests have adopted the constant virus-varying serum technique. The experimental considerations that recommend this procedure for most purposes have been cogently stated by Tyrrell and Horsfall.[48] In carrying out the test in this manner, serial dilutions of the serum are prepared, and each dilution is mixed with an equal volume of a virus suspension containing a fixed number of infecting doses for the tissue-culture system employed. After allowing the mixture to stand for a time, aliquots of each are inoculated into a number of cultures. Usually two or three cultures have been employed for each dilution of serum. In the case of the poliomyelitis viruses the cultures are examined at frequent intervals thereafter for evidence of cell destruction. The neutralizing titer has been taken as the highest dilution of serum that prevents the cytopathogenic action of these agents after a certain lapse of time. An example of how the techniques may be applied in following the development of antibodies after infection with poliomyelitis virus is afforded by the data included in TABLE 4. Tests were carried out on three specimens of sera, one of which was taken from a patient at an early stage of the disease, and two at later intervals. One hundred ID_{50} of the Brunhilde (Type I) strain were employed in the test, since a Type I virus was isolated in tissue culture from the feces of this individual. The antibody response is typical of the result encountered in most patients afflicted with the disease.[42]

Some factors involved in the tissue-culture neutralization test. Of those who have made use of the neutralization test in the study of poliomyelitis, Ledinko and her co-workers[27] have analyzed most extensively various factors that may influence the results. For example, in roller-tube cultures of monkey testis, they have found that a straight-line logarithmic relationship exists between the

TABLE 4

RESULTS OF TESTS FOR NEUTRALIZING ANTIBODY *vs.* TYPE I POLIOMYELITIS VIRUS IN SPECIMENS OF SERUM FROM A PATIENT WITH PARALYTIC POLIOMYELITIS

Serum	Cytopathogenic effect of virus in serum diluted				
	1/16*	1/32	1/64	1/128	1/256
Acute phase	+	nd	nd	nd	nd
Early conv.	0	+	+	+	+
Late conv.	0	0	0	0	+

* Final dilution of serum in virus-serum mixture before addition to roller-tube cultures of human uterine tissue. 0.1 ml. of each mixture was inoculated. Readings taken on 7th day.

serum titer and the quantity of virus neutralized. Since in this system the slope of the neutralization curve is approximately 1, it would appear that, for every tenfold increase in antibody, 10 times as much virus will be neutralized. This increase also means, of course, that the smaller the amount of virus used in the test, the greater will be its sensitivity in revealing small amounts of antibody. Although extreme sensitivity is not usually an absolute requirement in titrations of sera containing large quantities of antibody, it becomes essential in testing the sera of normal persons where its concentration may be low. Under such circumstances, unless the conditions of the test are adjusted to yield maximum sensitivity, erroneous conclusions may be drawn in respect to the immune state. So, for example, the emergence of antibody after inoculation of a vaccine might be interpreted as a primary response to the antigen, when in reality it might represent only a "booster" effect. Again, the results of epidemiologic studies carried out by means of the neutralization test and concerned with the inapparent attack rates of poliomyelitis might be misleading unless a procedure capable of revealing very small amounts of antibody is utilized. Accordingly, the determination of the neutralization curve by Ledinko and her associates is of importance, since it clearly indicates that the amount of virus used should be as small as possible.

There are limits, however, to the number of infective doses of virus that can be successfully employed; for if the virus inoculum is too small, certain of the control tubes to which virus alone has been added will not exhibit cytopathic changes, although a careful titration of infectivity may have previously been carried out. Furthermore, the use of extremely small doses of virus will unduly delay the outcome of the tests, since with minimal quantities the cytopathogenic effect may be delayed for as long as 10 or 11 days. Ledinko and her co-workers have therefore recommended 100 ID_{50} as a standard quantity of virus to be used in neutralization tests, and this dose has subsequently been adopted by other workers in the field. In repeated titrations, however, in which from 6 to 10 cultures of human uterus tissue have been inoculated with the same dilution of virus, we found that 30 ID_{50} uniformly causes cell destruction. Since smaller amounts have given irregular results, it would seem that about 30 ID_{50} represents the minimal useful quantity that can be employed.

Another way in which the sensitivity of the test might be increased is suggested by the results of experiments in which the addition of a thermolabile factor present in normal sera has enhanced the neutralizing effect with other virus-antibody systems.[49, 50] But attempts to demonstrate this phenomenon with poliomyelitis virus in tissue culture by Ledinko *et al*[27] have failed. On the other hand, in three experiments of our own in which twofold dilutions of serum were used instead of 10 as employed by Ledinko, a slight but consistent increase in the antibody titers was noted. This increase in antibody titer occurred in the presence of both fresh unheated serum and in heated monkey antiserum to which was added fresh normal guinea-pig serum. If future studies confirm these observations, the inclusion of unheated guinea-pig serum might be recommended in tests for neutralizing antibody as an aid in the attainment of maximal sensitivity.

One remaining technical point deserves remark before concluding this discussion of the neutralization test. It has been constantly observed by those employing tissue cultures in the assay of poliomyelitis antibody that, in the case of higher serum dilutions, the cytopathogenic action of the virus may be temporarily checked but eventually becomes manifested as incubation is continued. The endpoint of serum neutralizing capacity might accordingly appear as 1:1000 when readings are made on the fourth or fifth day and then decline to a lower value when the cultures are examined on the sixth or seventh day. After this time, in our experience at least, no change in titer usually takes place. Because of this tendency of the virus to "break through" the restraining influence of low concentrations of antibody, Ledinko and her associates have arbitrarily selected the fourth day for the final reading of the endpoint. We are inclined to think they are justified in so doing since there can be little doubt that antibody is responsible for this transitory inhibition of viral activity in the higher dilutions of serum. Yet it can be maintained that it would be more logical to take as the true endpoint that dilution of serum that leads to permanent neutralization of the virus. If this argument be accepted, however, accuracy would probably be sacrificed for the sake of pure reason.

In conclusion, I should hope that this imperfect sketch of the efforts of many investigators to fashion the tissue culture into a truly effective device for the production and assay of viruses and their antibodies will indicate that their efforts have finally met with success. Indeed I am confident the technique will take a permanent place, among those available to the virologist, alongside the experimental animal and that, in certain situations, it will even supplant the animal. Through the continuously widening application of the tissue culture, as procedure is simplified and accuracy increased, progress in this field should be rapid.

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DRUG ACTION ON CELLS *IN VITRO*

(Exhibition of a Motion Picture)*

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A sector of an untreated culture is shown to demonstrate the type of cultures mostly used in the experiments.[1] They are grown with the hanging-drop method in clotted medium. The material used is mesenchymal tissue of chicken embryos. The explants were transferred at least 15 times before being used for experimental purposes.

Drug action on cells *in vitro* is demonstrated in three groups: the first showing the formation of aqueous vacuoles in the cytoplasm; the second, the effect of some substances altering the oxygen uptake of cells; and the third, the effects of some mitotic poisons.

Vacuolization of the cytoplasm can be brought about either by the addition of ammonium salts or salts of organic amines to the medium, or by the addition of any substance splitting off ammonia during intracellular degradation.

To a fibroblast culture grown in a normal medium for 24 hours, ammonium carbonate is added in a concentration of 100 μg./ml. Ten minutes after the addition, vacuoles arise, increase in size, and flow together.

The second substance is adenosine degraded to hypoxanthine and ammonia. This culture was explanted with the addition of adenosine (1 mgm./ml.). An accessory effect of this substance is that the cell surface becomes less tight than in normal cells. Some cells show a bubbling of the cell surface without going into mitosis.

A third substance giving rise to vacuoles is adenosintriphosphate (ATP). The characteristic effect of ATP, when added in high dosages (3 mgm./ml.), can be observed. The cells are rigid. There is not only no movement of the cell surface but also no movement within the cells. One hour after the addition of ATP, the decomposition of this substance into AMP, inosinic acid, and ammonia begins and, after a short period of rigidity, the cells start to move again and vacuoles are formed.

In the second group (demonstrating the effects of changes in the oxygen consumption of cells), the first drug is pyocyanine. Pyocyanine increases the oxygen uptake of cells. The outgrowth of a normal untreated fibroblast culture is compared to that of a culture explanted with the addition of pyocyanine (12 μg./ml.). There is no difference in the cell migration. But the number of mitoses is different. A normal culture has an average of 2 to 3 per cent mitoses after 24 hours, while a culture treated with 12 μg./ml. pyocyanine has almost no mitoses at all. In several cultures, we found but one. In consequence, the central portion of these cultures becomes very thin. Twenty-four hours later, the zone of outgrowth becomes less dense and remains smaller compared to the controls.

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* This motion picture was shown at the Conference in connection with the rest of the papers published in this monograph. The accompanying description constitutes an important contribution to the subject and is therefore published in its entirety.

The influence of anaerobiosis on normal fibroblasts and sarcoma cells is next demonstrated. For this purpose, cultures are grown in a small chamber connected with a second chamber. In the latter, a small piece of yellow phosphorus is placed, thus consuming the oxygen of both chambers. When this experiment is made using a fibroblast culture already grown in the hanging drop for 24 hours, the cells are not able to adapt immediately to this environmental change. The medium is poor in glucose and, therefore, the energy-production by glycolysis is not sufficient to maintain the surface tonus. Fifteen minutes after the addition of the phosphorus, the cell-processes shorten up and become blunt. Later, the cells begin to push out pseudopodia and round up like cells in mitosis. These phenomena are reversible when the cultures are transferred to aerobic conditions.

In the same cell type, after the addition of phosphorus, under the same anaerobic conditions, but with the addition of 300 mgm./ml. glucose, the shift to glycolysis is facilitated and, therefore, the fibroblasts are able to maintain their spindle shape. Nevertheless, the tonus of the cell surface is reduced, one cell going into mitosis and pushing out pseudopodia of abnormal size.

This experiment was repeated with a culture of a benzopyrene sarcoma of the rat. First, a normal mitosis of these sarcoma cells is observed. Another culture of the same strain is subjected to anaerobiosis and no glucose is added. In contrast to normal fibroblasts, the sarcoma cells are able to adapt immediately to the withdrawal of oxygen, since they have a very pronounced capacity of glycolysis. Neither the type of mitosis nor the shape of the cells in interphase is altered in the way demonstrated on normal fibroblasts.

The effect of respiratory-poisons acting on mitochondria is next observed. The first drug is berberine. It could be demonstrated that berberine is being accumulated in the mitochondria.[2] The respiration of tissue cultures treated with berberine was measured in a Warburg apparatus and a decrease of the oxygen uptake was found beginning immediately after the addition of this drug. After 30 to 40 minutes, the minimum of cell respiration was reached. The glycolysis was not disturbed.[3]

To a fibroblast culture grown in a normal medium for 24 hours, berberine is added in a concentration of 150 μg./ml. Fifteen minutes after the addition of the substance, the surfaces of all cells in the picture are in motion. The cell processes shorten. First, there is a slight rippling and frothing, and then short pseudopodia are pushed out. Later, a short period of almost no motion occurs, followed by very intense bubbling of the cell surface. With this dosage, the cells continue to move for hours.

The same drug is then added to an epithelial culture. First, a normal untreated culture of cornea epithelium of the rabbit is shown in order to demonstrate the normal way of outgrowth of epithelium in the form of a membrane. Since it is difficult to observe isolated cells while growing in a membrane, we chose for the berberine-experiment an epithelial cord where the border of many cells can easily be observed. The dosage of berberine is lower than in the experiment with the fibroblasts (50 μg./ml.). The cord migrates slowly and small pseudopodia are distinctly visible, pushed out at the border of every cell. That means we have the same phenomenon as demonstrated on fibroblasts, but less intense, which is in agreement with the smaller dosage employed.

Another respiratory-poison which is effective through its adsorption by the mitochondria is the dye Victoria blue.[4,5] The mitochondria stain very intensively blue and stick to the nucleus. The marginal part of the cytoplasm is free of mitochondria and starts moving. Both the intensity of the effect and the time interval between the application of the drug and the beginning of cell movement depend on the concentration of the drug added. The effect of a rather high dosage (20 μg./ml.) is demonstrated, showing the diffusion of the dye from one side of the clot towards the culture. The cells start to move as soon as they have accumulated the dye. The cells round up and begin to push out big pseudopodia. These cells are irreversibly damaged and blow up later.

The third group of drugs is that of mitotic poisons. The first substance is acriflavine which reacts with the nucleic acids. It produces the well-known phenomena of the sticking and clumping of the chromosomes during mitosis. Fibroblast cultures were grown with the addition of 1 μg./ml. acriflavine for 24 hours. A mitosis is shown with the sticking of two pairs of chromosomes thus forming a chromosome bridge. The cleavage furrow is formed but the daughter cells are not able to divide completely and remain connected by a thread of chromatin. Another cell in mitosis shows the clumping of the chromosomes which are not able to separate. Nevertheless, the cell pushes out pseudopodia like a cell in anaphase.

The next substance is colchicine, inhibiting the action of the cell spindle. The culture was grown with the addition of 0.02 μg./ml. of colchicine for 24 hours. With such a high dosage we have (after 24 hours) about 30 per cent of the cells arrested in metaphase, the chromosomes being either clumped together or scattered over the cell. The movement of the cell surface is different from that observed in cells treated with acriflavin. There is no bubbling but more extensive movements of the entire cell with a smooth surface, thus forming the well-known bizarre shapes of colchicine-treated cells.

Desoxycorticosterone acetate acts as a synergist of colchicine. A culture is demonstrated, grown with the smaller dosage of 0.01 μg./ml. of colchicine, but with the addition of a small crystal of desoxycorticosterone acetate in some distance of the culture. The cell motion is slower and the cell surface less tight, the surface tonus being even more reduced.

The last substance is adrenaline, acting when transformed to adrenochrome. The colorless substance is added to the cultures when transferred. After 24 hours, the color of the yellowish clot has changed to a reddish brown. The same effects as obtained by the addition of adrenaline can be produced by adding pure adrenochrome.

Finally, the action of a threshold dosage of adrenaline (90 μg./ml.) is demonstrated. As a result of these threshold dosages, we have a slight increase of the number of mitoses. We find normal mitoses as well as both arrested mitoses and mitoses with normal nuclear division but failure of cytoplasmic division thus forming binucleate cells. These three kinds of division—normal division, mitotic arrest, and formation of a binucleate cell—are shown simultaneously.

The valuable assistance of Doctor A. Schleich and of A. Mayer is gratefully acknowledged.

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ACTIVITIES AND RESPONSES OF LIVING CELLS AND THEIR COMPONENTS AS RECORDED BY CINEPHASE MICROSCOPY AND ELECTRON MICROSCOPY[*]

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Some of the very remarkable properties of the protoplasm of living cells must be related to the basic structure of the fibrous gels of which it is composed. The free and purposeful movement of the organized structures and of different cell components, apparently differing in function, can now be visualized and studied quite well with advanced methods of physical optics. By the proper use of phase optics and time-lapse photomicrography,[1] a remarkable comparison of the living cell's composition and structure can be made with similar but fixed structures examined in the same or an homologous cell, at much higher powers, under the electron microscope.[2, 3, 4] A series of static images and short motion picture scenes were used to illustrate some of the similarities and differences in the structural organization and the behavior of various components of the tumorous cell strain of the rat (T-333) derived *in vitro* from a normal fibroblast strain 14p.[5] Cinephase studies were also made of the 23-year-old strain of human chondromyxosarcoma, strain D-1 Re; of the 15-year-old human fibrosarcoma, strain A.Fi.; and of the 3-year-old strain of human epidermoid carcinoma of the cervix, strain HeLa, when grown in thin tissue-culture slides.

These motion pictures show clearly the structural transformations of the very active superficial plasmagel layer of these cells. Coarse fibrous processes and fine microfibrils, especially numerous in the normal strain 14p cells, reveal their long filamentous character as shown in PLATE I, FIGURES 1 and 2, and reveal their narrow dimensions (some about 0.2 microns in diameter). These microfibrils show their remarkable ability to grow rapidly in crystalline fashion, then stiffen and often break off, or even "deliquesce" into the receding less polymerized or solated base from which they originally appeared. In PLATE I, FIGURE 1, the upper edge of this thinly spread out normal rat fibroblast shows three distinct and two fainter microfibrils, all of which began as tiny protrusions from the cell edge or from the surface and within a few minutes became greatly elongated. Some of them may become several times longer than those shown here.[6] Such an image can be compared directly with the microfibrils seen in thin sections with electron microscopy as shown in PLATE 1, FIGURE 2, where one long and two shorter ones may be seen projecting from the plasmagel membrane. In some cases the microfibril may be seen to be composed of two components or represented as two states of gelated protoplasm, a stiff fiber component and a softer beaded component. It may often be seen to recede toward the cell membrane like a tiny droplet of water falling down a single spider-web fiber or the entire fibril may "deliquesce" and fuse with plasmagel membrane. In the electron micrographs made so far today, we have not detected any basic periodicity in these remarkable microfibrils. They may be seen on a variety of living cells examined by phase microscopy. Some of them are shown in PLATE VI, FIGURES 21, 22, and 23, both as surface protrusions and as web-like continuations clearly depicted in PLATE VI, FIGURE 22, at the left-hand border of the human fibrosarcoma cell.

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Other types of protoplasmic processes were also the objects of study in the motion picture scenes taken with phase-contrast microscopy of a variety of cells. These types are best illustrated, for example, by the ebb and flow of the very thin membranous pseudopodia (ruffles or folds) derived from the superficial plasmagel layer or "ectoplasmic zone" referred to by others. In PLATE V, FIGURES 15 through 20, the free border and somewhat transparent surface shows many such folds which, in some instances, are linear in their visual display and may be confused with the more filamentous mitochondria seen beneath them.[13] These folds accomplish the initial stages of pinocytosis[8, 9] and will be described later. Here again, images of the living-cell surface activities can be compared with electron micrographs in comparable regions and thus permit the detailed pattern to be restudied again and again by the use of motion pictures which help greatly to establish and orient clearly the nature of the cell processes. By these means, we get a clear interpretation of the cell's ultrastructure at any point in question. This region of the cell defines its contact with its immediate exterior milieu and apparently determines what is engulfed.

In PLATE I, FIGURE 3, we see the outer limiting border or plasmagel membrane thinly spread out but, at this moment, having fewer microfibrils. Membranous components, such as those shown at regions 1 and 2 may be interpreted as precursors of a stage when pinocytosis begins. Regions 1 and 2 of this cell can easily trap droplets of the culture fluid and rapidly incorporate them in the streaming hyaloplasm. In PLATE I, FIGURE 4, may be seen two protrusions from a thin section of a cell of this fibroblast strain, 14p. These may be interpreted as having already trapped two masses in regions 1 and 2. In less than a minute, droplets of this size have been observed to move inward, diminish greatly in size, and disappear from view or change their phase characteristics. Pinocytosis, however, is not prominent in this strain, as may be judged by an examination of the juxtanuclear region shown in FIGURE 5, where only a few old inclusion droplets are found, some of them already associated or combined with pre-existing mitochondria. In FIGURE 6 is seen a thin section of a comparable region in another 14p cell but with a greatly folded nuclear membrane. The two broad streaks stretching across the nucleus, shown in FIGURE 5, are superficial folds in the cell membrane. Indentations in the nuclear membrane in FIGURE 6 are not interpreted to be artifacts, since living nuclei in many different kinds of cells show indentations of their nuclear membranes and, as well, protrusions. The darker round bodies seen in the electron micrograph, FIGURE 6, are apparently inclusion droplets comparable to those seen in the region of the nucleus in FIGURES 1 and 5, PLATE I. The elongated structures and many of the oval ones represent longitudinal or tangential sections of mitochondria and many of the darker masses have been interpreted as old inclusion droplets. One can readily appreciate a variety of shapes of mitochondrial images obtained in an ultrathin section. By comparing the images of FIGURE 6 with the intact living images of FIGURES 1, 3, and 5, a careful examination and interpretation of the normal living cell's structure and behavior in tissue culture has allowed us to make some comparisons with strain T-333 cells of tumorous origin.

When a comparison is made between the thin normal prototype strain 14p of rat fibroblasts, with its tumor cell derivative of *in vitro* origin, and the static images of PLATE IV, FIGURES 15 and 16, and the motion picture sequences of PLATE II, FIGURES 7 and 8, and PLATE III, FIGURES 9 to 12, one readily realizes that such a comparison will show malignant cells to be (1) thicker; (2) to show a prominent ring of inclusion droplets distributed around the nucleus and juxtanuclear area; (3) to have a large juxtanuclear area so ridged as to bend the large polyploid nucleus found in many malignant cells (PLATE II, FIGURE 8); (4) to manifest increased feeding habits through abundant pinocytosis; and (5) to have a larger centrosphere (PLATE IV, FIGURE 14) in the region shown by the arrow. Also, the tumor-cell derivatives show less cohesion of the cells of their colonies and fewer microfibrils.

The rates of pinocytosis in strain T-333 may be appreciated by following the rate of transport of two newly engulfed inclusion droplets (PLATE III, FIGURE 9) which within two minutes are reduced to a smaller sphere (FIGURE 10) and within three minutes become much smaller and are already incorporated in the major ring of droplets around the nuclear area. In this case, there was no change in the index of refraction, since no change in hue has occurred. In many cells such an inclusion droplet would become transformed into a tiny bright particle by the time it had reached the nuclear zone. It may be interesting to note that many inclusion droplets are not content to follow a straight path to the juxtanuclear zone but will race around the nucleus to come to rest for a time in a specific juxtanuclear area. This area, the giant centrosphere, is commonly found indenting the nuclear membrane as shown in PLATE II, FIGURE 8. Pinocytosis may be clearly observed at one focal level with phase microscopy (PLATE II, FIGURE 7) or the final aggregation of the particles revealed at another focal plane (PLATE II, FIGURE 8) when the large kidney-shaped polyploid nucleus is brought into focus.

Repeated examinations reveal the important role of the streaming hyaloplasm in sweeping these inclusion droplets to the cytocenter, during which time they become greatly reduced in size. We have interpreted these changes as eventuating in the production of either beaded or filamentous-appearing mitochondria, some of which are composed of beads of various sizes as seen in PLATE III, FIGURES 9, 10, and 11, region 2. In fact, most of the mitochondria seen in FIGURES 9, 10, and 11, PLATE III, show a beaded structure, and in the motion-picture records move more or less independently of one another or pass by each other, up and down the well delineated, fibrous living partitions seen most clearly in FIGURE 12, PLATE III. One must examine repeatedly the excellent motion-picture records in order to comprehend fully the value of these records in establishing the nature of the endocellular traffic of these cellular components. Do these images and the course of events that can be easily followed justify the conclusion that mitochondria represent the fusion of old, and sometimes of young inclusion droplets with pre-existing mitochondria, or of a smaller precursor, the ultrachondriome?[12] This description of the visual display of living events, as seen in motion pictures, and of others to follow, have led us to consider this interpretation as one of the chief modes of mitochondrial growth in these cultured cells.

A more dramatic display of the process of pinocytosis and the relation of the inclusion droplets produced to the mitochondria are beautifully illustrated in the samples of motion picture records shown in PLATE V. Here the active membranous pseudopodia, FIGURE 15, shown at 3:10, regions 1 and 2, show newly-trapped droplets, and within two minutes, FIGURE 16, the droplets are already smaller and now spherical. At about 3:15, they have moved inward, have changed slightly in hue, and now are still smaller. Droplet No. 1 has already become grey and thus reveals a change in its refractive index. In less than a minute, droplet No. 2 has become grey; whereas No. 1 droplet becomes smaller and whiter. A minute later both droplets are still smaller and brighter. At the 15-minute period, FIGURE 20, both droplets have now become of the same bright hue and have moved inward considerably. Another droplet, No. 3, during this 15-minute interval, is reduced greatly in size without much shift in position but with some change in hue.

The more dramatic event of this visual display of endocellular traffic can be seen by comparing FIGURES 17 to 20 in an interval of about 10 minutes. In this series, a large triangular inclusion mass, No. 4, is trapped, and within less than a minute it appears as a smaller spherical inclusion droplet which, within another minute, begins to show evidences of change in hue with reduction in size. Near the end of the ten-minute period, FIGURE 20, the total reduction in volume can be estimated to be one tenth of the original mass and now shows a change in index of refraction, a whitish grey hue. Doctor Lewis described this process,[8, 9] emphasized the importance of it as a means of incorporating proteinogenous materials into cells, and described its similarity to phagocytosis.

In some cells a more intimate relationship of inclusion vacuoles and mitochondria may be revealed from carefully selected motion pictures, as shown in PLATES VI and VII. Here may be seen the remarkable effects of localized hyaloplasmic streaming on inclusion droplets and mitochondria which may engage one another during the droplet transport inward. In thinned out regions of the human fibrosarcoma cell from strain A.Fi., seen in PLATE VI, FIGURES 21 through 23, the forces acting upon very small inclusion droplet No. 1, on the mitochondrion No. 2 is revealed by the great displacement which occurs in this mitochondrion when the inclusion droplet engages it and produces a long loop as shown in FIGURE 22, just two minutes after their first contact. In another two minutes, the inclusion vacuole succeeds in tearing No. 2 mitochrondrion into two components, shown in FIGURE 23, and now has moved farther inward toward the nuclear zone. During this four-minute interval, it has become reduced in size to about half of its volume but without change in hue. One has reason to believe that by the time it gets to the juxtanuclear area it will become a tiny white droplet similar in size to any of those seen within the beaded mitochondrial chains or found in association with mitochondria.

This great reduction in size has been followed in other motion-picture scenes, and the fusion of these small inclusion droplets with pre-existing mitochondria is well established. In the earlier phases of their transport, the new unconcentrated inclusion droplets do not appear to fuse with the mitochondria, yet later, after considerable loss of fluid, their characteristics seem to be more suitable for mitochondrial incorporation. It is entirely likely that mitochondria can be formed from these droplets directly, as described above for cells of strain 14p and T-333. In some scenes, newly ingested inclusion droplets may disappear completely. It is quite likely they are discharged from the cell since one sees no evidence of their incorporation with other organelles.

A similar analysis has been made of mitochondrial distortion in a remarkable series illustrated in PLATE VII of a thinned out portion of a HeLa strain cell. Within an eight-minute period, inclusion droplet No. 1 engages mitochondrion No. 2, seen in FIGURES 24 through 26, and succeeds in breaking it within three minutes, as shown at 4:27, in FIGURE 3. At this time, it has moved a considerable distance and has now engaged a second mitochondrion, No. 3, and within two minutes (FIGURES 27 and 28) it stretches mitochondrion No. 3 to form two broken fragments No. 3a and No. 3b. At the six-minute interval, FIGURE 28, it has already engaged a third mitochondrion No. 4 and may be seen to move still farther on its way toward the juxtanuclear mass. These events reveal the dramatic movement of such components within 10 minutes. This remarkable process is not easily seen in regions with greatly conflicting streams of flow, especially in the thicker regions of cells. The basic mechanism of transport is still a subject of conjecture. Is it a simple folding and unfolding of protein monolayers? When cells such as these are examined at lower power, one is impressed with the overall character of the hyaloplasmic streaming which tends to sweep many particles from the periphery to the nuclear zone and out again.

Dramatic changes in shape of mitochondria have been described by Frederic and Chévremont.[10, 11] We have also seen such changes in the mitochondria of several cells that we have examined. The predominant changes in shape of mitochondria reported here would appear to be related more to the effects of local hyaloplasmic streaming than to any other process of which we have knowledge. The presence of the inclusion droplet is not necessary, since some mitochondria have been shown to be greatly distorted by local stream flow, even in the same path as that traversed by a droplet and having an equal stretching effect on the mitochondrion that is being observed.

A series of important events initiated by the ever-changing plasmagel membrane of both normal and malignant cells has been revealed through a series of motion picture recordings which depict its remarkable ability to produce exceedingly fine microfibrils or membranous pseudopodia. The latter may be shown to trap droplets of the surrounding medium as it passes them inward to the streaming hyaloplasm. The character of some of these cellular components are compared in the living state with fixed images seen in thin sections and viewed under the electron microscope. This most dramatic process is revealed be an examination of thinly spread out cells with cinephase microscopy, whereby inclusion droplets may be seen to pass to the juxtanuclear area and, in some cases, to stretch greatly and to distort the mitochondria which get in their way, and even to break them. These same particles, derived from the medium, become greatly reduced in size to a point where they become indistinguishable from the particles which make up beaded mitochondria. We have established the association of these transformed and greatly concentrated and reduced droplets with mitochondria, and have observed their fusion with them. The evidence indicates that mitochondria may be produced directly by the formation of chains of these inclusion droplets. In one instance, we have observed the transformation of a large concentrated inclusion droplet into a long filamentous mitochondrion. In degenerating cells, the opposite may be seen to occur frequently, that is, the formation of cystic mitochondria from filamentous ones. These comparative studies point to the need for a more intimate evaluation of the ultrastructure of inclusion droplets at various stages of their incorporation into the cell's basic components. Hyaloplasmic streaming would appear to be the basic driving force in this dramatic display of the endoplasmic traffic and it may, indeed, account for nuclear rotation as well. The process of microfibril formation, which in many ways resembles the growth of crystals, is yet to be revealed, but it may still be allocated to the concept of the folding and unfolding of protein monolayers as the primary mechanism involved.

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somatique. Etudiée dans des cellules vivantes cultivées in vitro par microscopie et microcinematographie en contraste de phase (2^{eme} partie). Arch. biol. (Liége). **63:** 259–277.

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PLATE I

FIGURES 1, 3, and 5 show various regions of the 14p strain of normal rat fibroblast, now in continuous cultures over 15 years, with bright phase oil immersion. Magnification, ×1000. The electron micrograph images in FIGURES 2, 4, and 6 show comparable regions of similar cells of this strain from thin sections. Veronal buffered osmic acid fixation was used. Magnification ×7500.

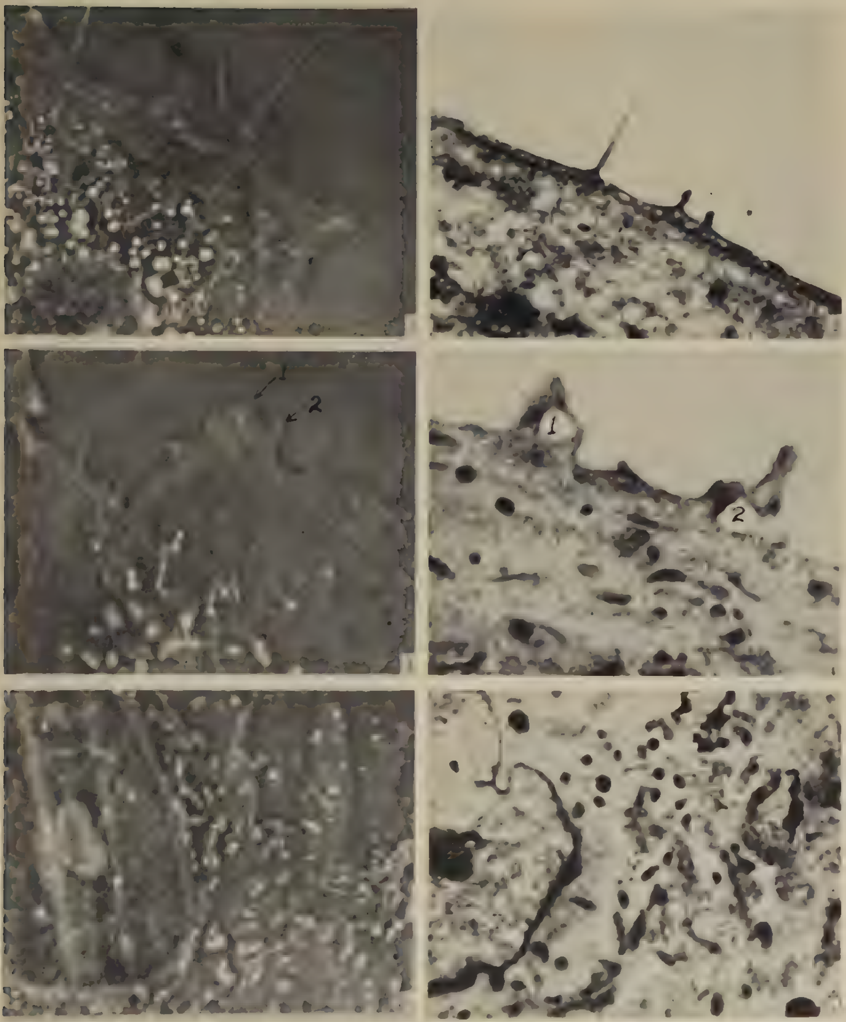
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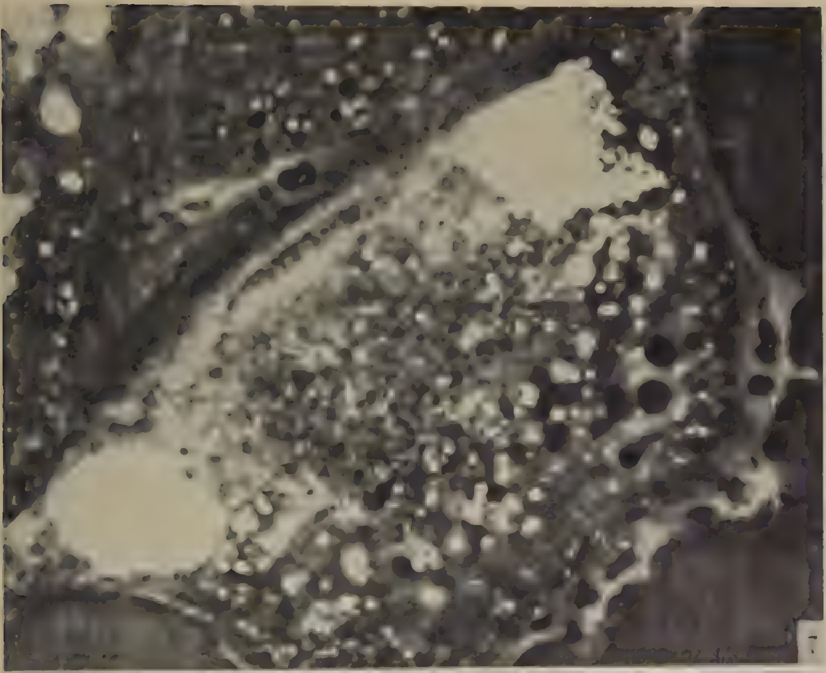
PLATE II

Bright phase contrast images of strain T-333 fibrosarcoma cells of the rat of in vitro origin. FIGURE 7 shows a focal plane with pinocytosis clearly defined and with dark inclusion droplets at the periphery and smaller bright inclusion droplets covering the nuclear region. FIGURE 8 shows another focal plane revealing the kidney-shaped polyploid nucleus and juxtanuclear area. Inclusion droplets are now out of focus. Living image. Magnification ×2100.



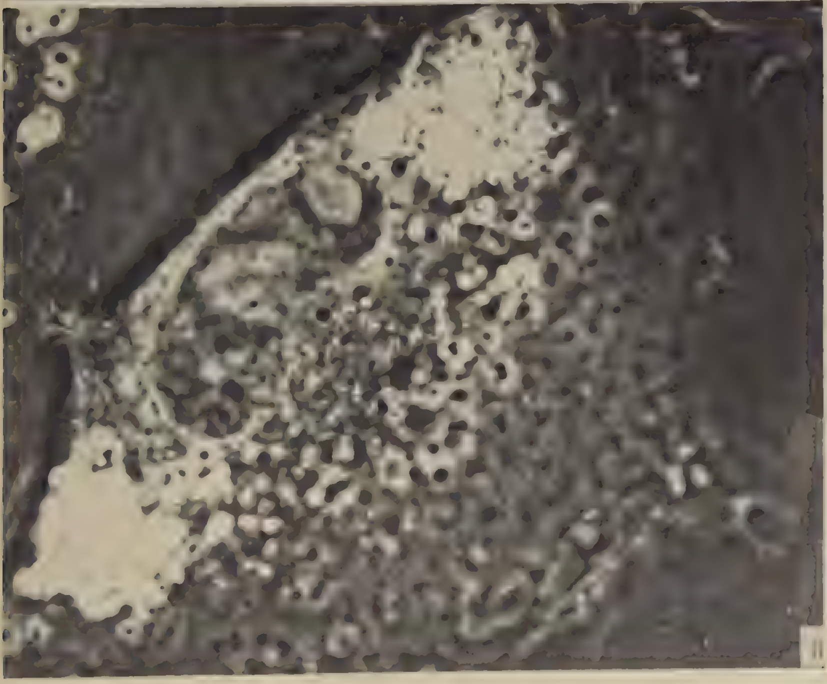
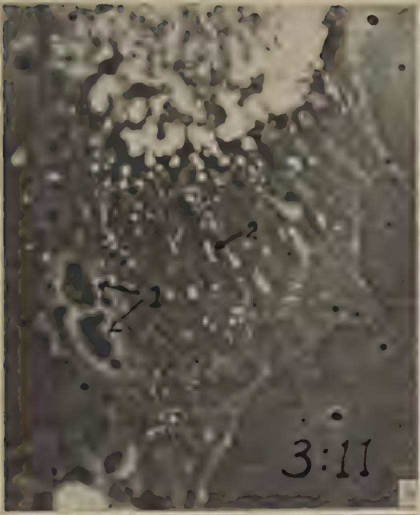

11

PLATE III

FIGURES 9, 10, and 11 show a thinly stretched out region of strain T-333 fibrosarcoma of the rat of in vitro origin. In FIGURE 9, region 1, are seen two newly engulfed inclusion vacuoles. Within five minutes (FIGURES 2 and 3) this mass passes to the juxtanuclear area with a reduction in size. In region 2, FIGURES 9, 10, and 11, beaded mitochondria are seen to be composed of varying numbers of beads of different sizes within the five min. period. Similar rapid shifts in the number of beads are also prominently displayed in all other areas and especially well in FIGURE 4, which illustrates very clearly the sharply delineated pathways which these mitochondria follow in moving rapidly from place to place. Living images with bright phase contrast. Magnification ×960.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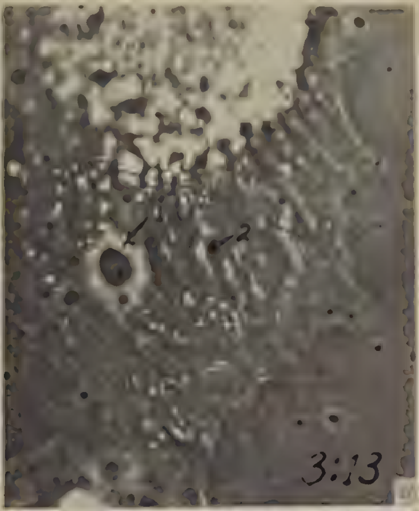
2
3:11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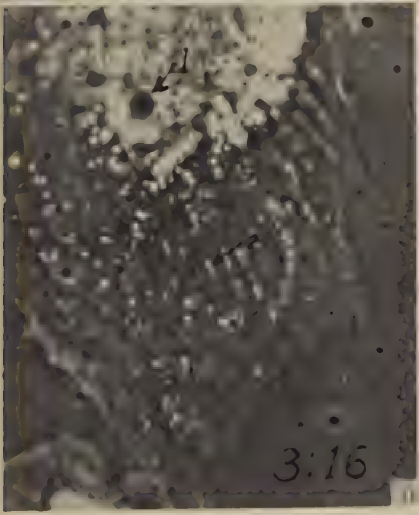
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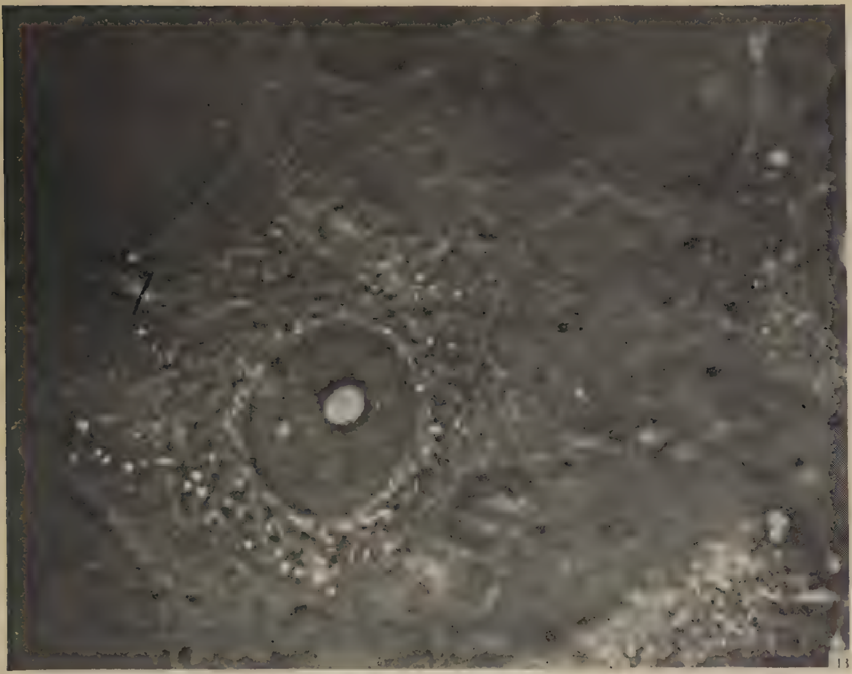
3:16

PLATE IV

FIGURE 13 shows a living image of the rat normal fibroblast strain 14p. Inclusion droplets are not numerous. FIGURE 14 shows a living image of tumorous strain T-333 of *in vitro* origin. The large polyploid nucleus is surrounded by a ring of inclusion droplets which also enclose the giant centrosphere; these cells are usually much larger and thicker than their specific normal prototype cell 14p (PLATE IV, FIGURE 1) from which they were derived *in vitro*. Magnification ×1500.



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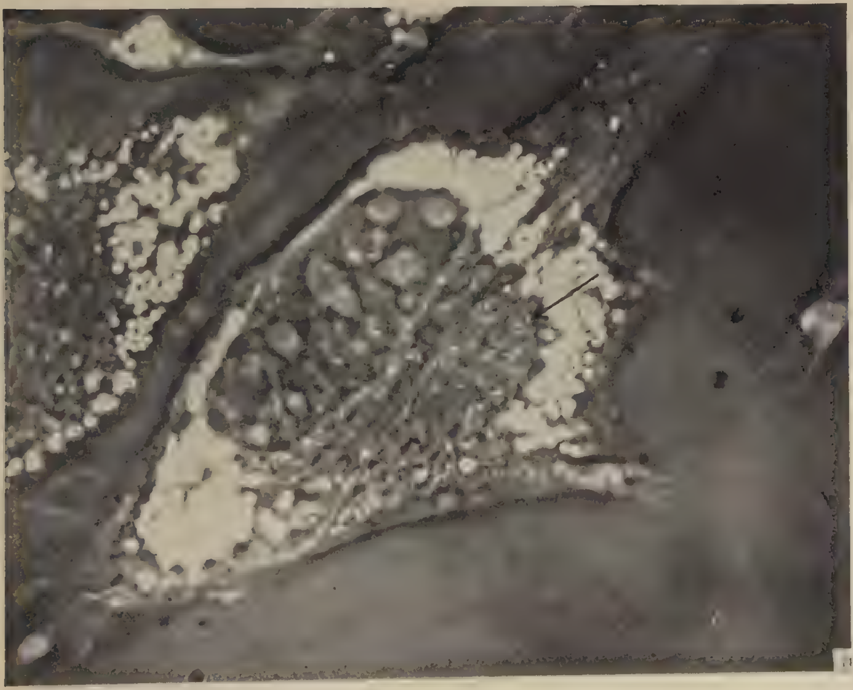
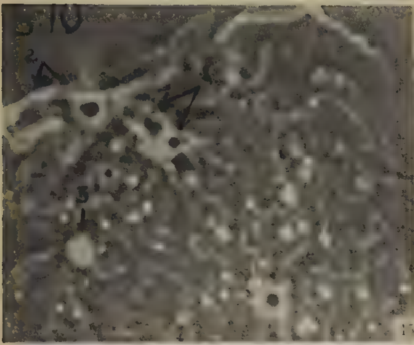


14

PLATE V

FIGURES 15 to 20 show a small region of a cell of the human chondromyxosarcoma strain D-1Re. In the total 15-minute interval, newly engulfed inclusion droplets in regions 1, 2, and 3 may be seen to be progressively reduced in size with a change in refractive index and visual display. Living cell image in the bright phase contrast. Magnification ×750.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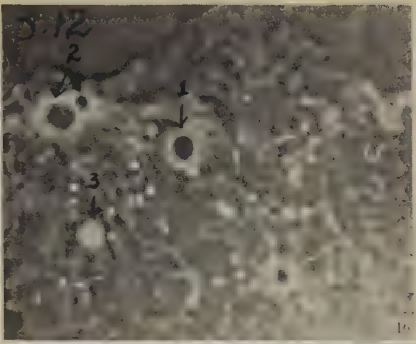
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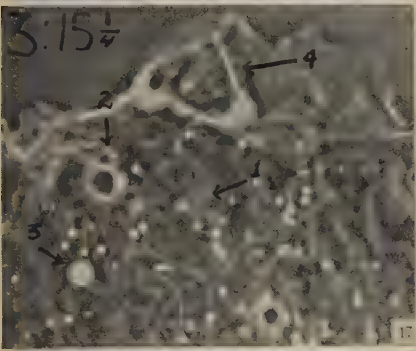
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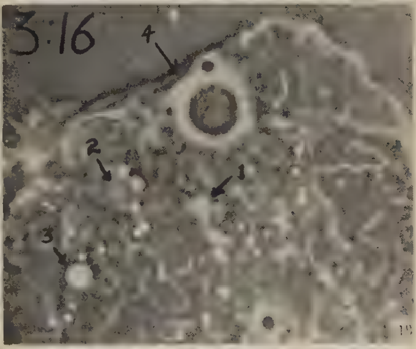
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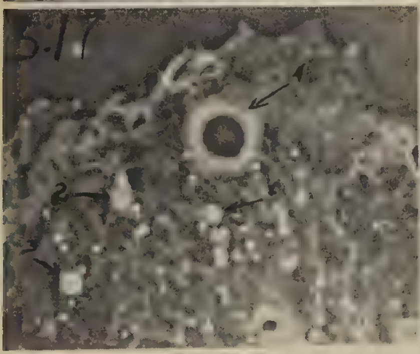
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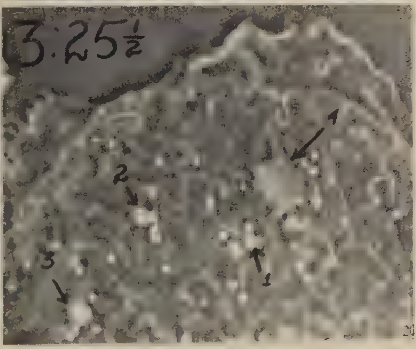
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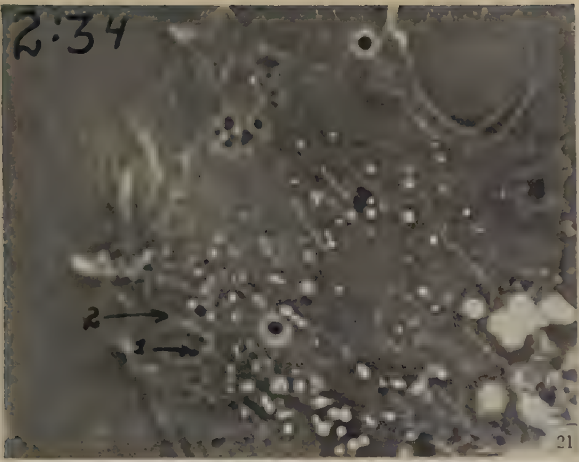
PLATE VI

FIGURES 21, 22, and 23 show a thin region of the human fibrosarcoma strain A.Fi. with bright phase contrast. The new inclusion droplet No. 1 stretches mitochondrion No. 2 as it passes inwards during a two minute interval. Within the next two min. it breaks the mitochondrion as shown in FIGURE 23. Living cell image. Magnification ×900.



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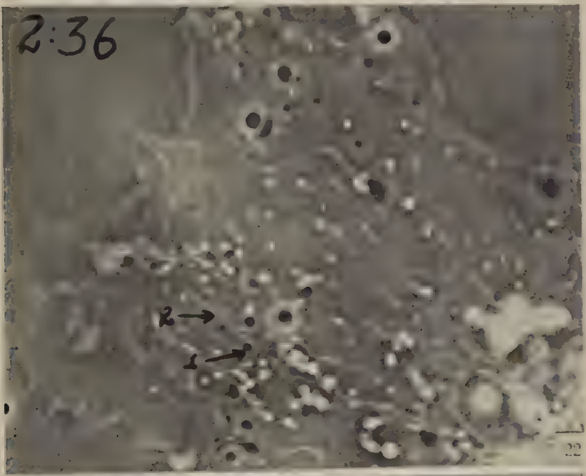

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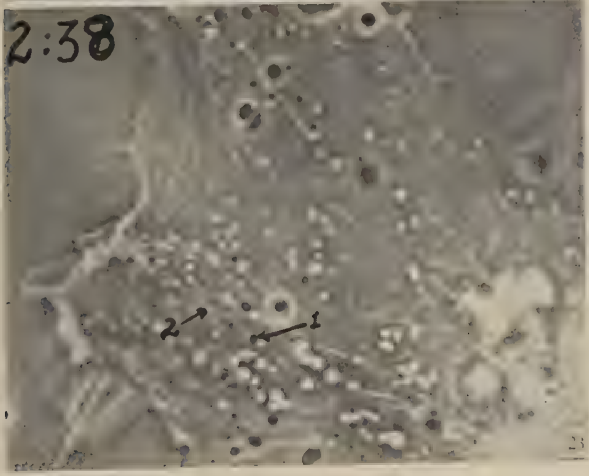
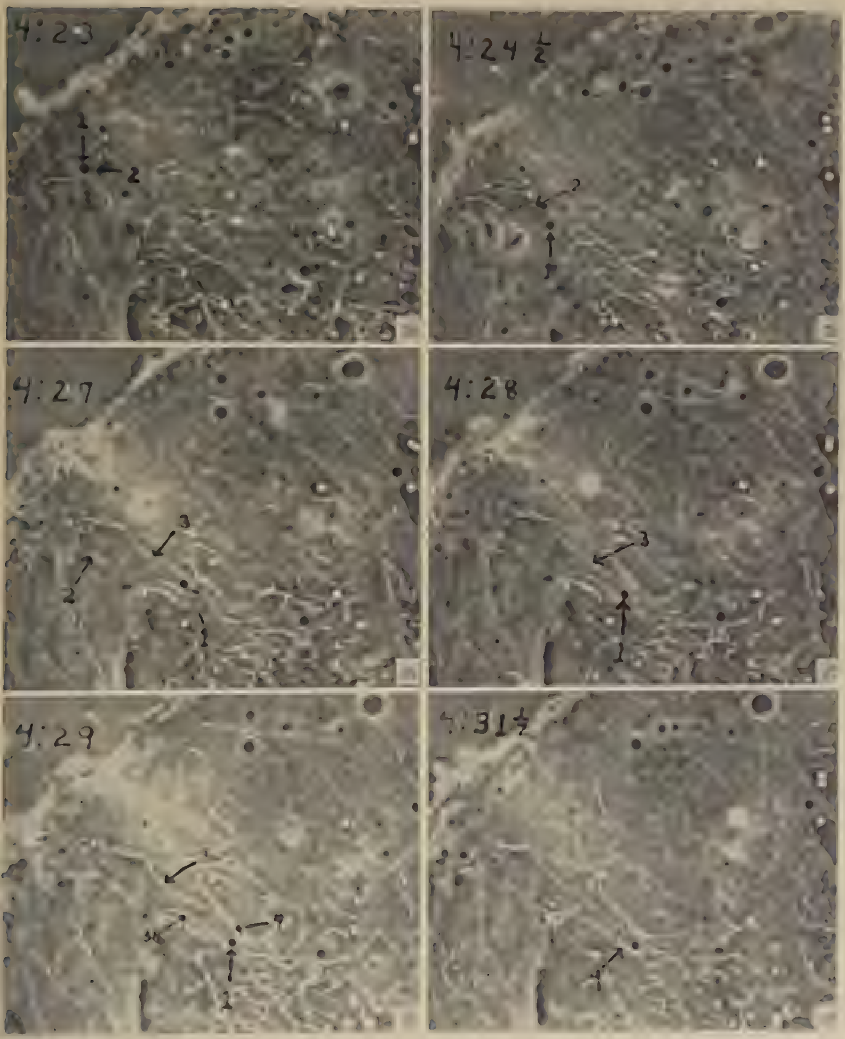


23

PLATE VII

FIGURES 24 to 29 show a small region of a cell of the human epidermoid carcinoma of the cervix, strain HeLa. During a 9-minute interval, the new inclusion droplet No. 1 breaks through three mitochondria Nos. 25 to 27 as it passes inwards with a reduction in size. Living cell image. Magnification ×1500.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4:29
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4:31½

Part II. Special Assay Technique: Comparison Between *in Vitro* and *in Vivo* Results

SHORT TERM CULTURES FOR DRUG ASSAYS: GENERAL CONSIDERATIONS*

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The application of tissue-culture methods to pharmacological investigation may well be considered from the standpoint of the special scientific problems of pharmacology and the help that can be brought to them by use of tissue-culture technique. Pharmacology is the study of the action of chemicals on living things. In a broad scientific way, it matters not whether the action is harmful or beneficial, or whether the living material is plant or animal. The peculiar problems of a scientific nature which are included in the scope of pharmacology are (1) the dose-effect relation; (2) time concentration relationships; (3) the absorption, distribution, metabolism, and fate of chemicals introduced into living tissue; (4) the relationship between chemical constitution and biological action; and (5) the mechanisms by which chemical molecules introduced into living material react with the molecules comprising the cells of living things.

The advantages of tissue-culture methods in pharmacology are (1) more precise control over the experimental conditions than is possible with the use of complex mammalian organisms, with exact control of temperature, solutions used, time factors, and recording; (2) strictly uniform living material, of which many samples can be taken from the same identical source, so that the results obtained may be subjected to sharp statistical analysis; (3) relative simplicity of experimental equipment and methodology; (4) the possibility of accurate observation at a cellular level; and (5) the possibility of recording the results in the dynamic relationships of time change.

The disadvantages of the use of tissue culture methods in pharmacology are (1) little indication of the effects of chemicals on functional activity of complex organisms such as mammals; (2) limitations of observation of effects of chemicals on living things to modifications of cellular activity and changes in rate and character of growth; and (3) the loss of "gestalt" in relation to the concept of the mammalian organism as a whole.

Tissue culture methods may be profitably used in pharmacology for rapid screening of the abundance of new chemical agents which are continually being developed by organic chemists. This screening can be undertaken for such general matters as differential toxicity and specific organ susceptibility, or it may be limited to specific types of action such as nerve depression or skin injury. With the introduction of quantitative methods in tissue culture, whereby the growing material can be placed in a calibrated chamber for observation under

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exposure to specific concentrations of various chemicals, one may systematically study quantitative metabolic changes as correlated with structural effects, or the rates of utilization of essential metabolites, or interferences with normal metabolism under the influence of antimetabolites. One may investigate toxicity reactions with respect to the lowest concentration that gives evidence of any interference with growth at all, or to the concentration which will inhibit growth completely. In such a screening study, one may note the evidence of toxic injury on the growing cells.

This type of toxicity screening may be extended to chemotherapeutic screening, where the parasitic organisms involved may be grown directly in the tissues concerned. This type of screening may be undertaken for protozoan invaders, bacterial organisms, or for viruses. Well suited is this arrangement for the systematic screening of new antibiotics. It is also being successfully adapted to the study of antiviral substances.

Of considerable practical importance is the variation in action of chemicals on the various organs of the complex mammalian body. This variation can be conveniently studied by tissue culture techniques where the various organs may be separately grown. Under these circumstances, the differential organ susceptibility to various chemicals may be specifically estimated. These estimates may assist materially in learning in advance the possibility of unsatisfactory side actions of drugs that may be developed for some specific organ effect.

Quite practically, the tissue-culture methods may be applied for systematic screening of such drugs as antihistaminics. The development of successful methods for growing sensitized human nasal mucous membrane makes it possible to evaluate various antihistaminics that may be proposed for protection against various types of allergy.

Similar screening studies may be made of the action of cardiac glucosides on beating heart fragments. This possibility has not yet been adequately explored, but it offers many promising opportunities for fruitful observation. Similar screening may be done for spasmolytic agents, using tissue culture of smooth muscle.

Nerve conduction in tissue culture might be investigated, so that drugs might be effectively screened for nerve-depressant action. The possibility of injurious effects of drugs on the brain can be directly studied by tissue-culture methods in so far as nonneural structures are concerned. Tissue-culture techniques offer excellent possibilities for the testing of drug action on the skin, whether from the standpoint of irritation and injury or from the viewpoint of promoting skin healing.

Nutritional requirements of various tissues of the body can be appropriately examined in tissue culture. Such observations could include various pharmacological aspects of vitamins, amino acids, and mineral dietary constituents. Tissue-culture methods are particularly well suited for an investigation of the effects of trace elements. The localization of chemicals in cells, tissues, or organs may be studied by tissue-culture methods, with possible appreciation in diagnosis if radio-opaque chemicals can be formed which are specifically localized. Using radioactive isotopes, such studies may be of great value in learning where chemicals act in cells.

One of the most important aspects of tissue-culture technique is recording by time-lapse movies, using phase-contrast microscopy. This study gives an extraordinarily vivid, dynamic impression of the living process. Gone forever, after viewing motion pictures recording cellular activity in tissue culture by time-lapse movies, is the notion of the fixed rigidity of cells. It is now possible to study the effects of drugs on nuclear rotation, on mitochondrial activity, on vacuole formation, and on the imbibition of surrounding materials into the cell. From such observations may come important data affecting theories of absorption, excretion, permeability, and metabolism. By tissue-culture methods, one may also study the effects of drugs on cellular division and maturation, on macrophage activity, and on the pulsating action of cardiac cells, on sensitized mucosa, on smooth muscle and glandular function, and on oligodendroglia in the human brain.

Drug Toxicity in Tissue Culture

Many drugs have been studied with a variety of experimental procedures in the vast literature of tissue-culture work. A comprehensive digest of the data pertinent to pharmacology has been undertaken by Rolf Meier and his associates.

In reviewing the extraordinarily comprehensive *Bibliography of The Research in Tissue Culture*, compiled so painstakingly by Margaret R. Murray and Gertrude Kopech, one is impressed by the amazing number of references to drug action studied by tissue-culture methods. These references extend from abstinence phenomena in relation to addiction, and from such drugs as azonaphthene compounds (carcinogens, for example) to zinc compounds and zonite. On acetic acid and its derivatives there are 41 references. On drug addiction studied in tissue culture there are listed 28 reports. There are 48 references to tissue-culture studies of cardiovascular drugs. Unfortunately, the classification for the drugs is somewhat confusing. Sulfanilamides and arsphenamines are accordingly included among antibiotics. Excellent cross referencing, however, makes it possible to find source material on the tissue-culture effects of practically all types of drugs and poisons. Indeed, there are 57 pages of references to the poisonous action of chemicals as observed by tissue culture, totaling perhaps 1800 publications.

The preliminary data presented in this report on the tissue-culture effects of certain selected drugs are limited to the definition of their toxicity on certain standard test tissues. Many obvious questions will have to remain unanswered at this time.

The chemicals employed in the results given here reflect research interests by many clinical and laboratory groups. The findings therefore largely represent the by-products of other efforts. In spite of incompleteness, the data on the drugs listed are offered as a "first approximation," which may be useful to others, and as a beginning for a table of toxicity data which might become useful in the standardization of information on drug-cell relations.

TABLE SHOWING TISSUE-CULTURE TOXICITY FOR VARIOUS COMPOUNDS AND PREPARATIONS

No.	Substance	Identity	M.W.	Test tissue	Concentration in γ/ml. of medium	
					Outgrowth stops	Detectable injury
1	Acetone	(Solvent)	58.08	Cord	>250,000	15,000–30,000
				Heart	>250,000	15,000–30 000
				Spleen	>250,000	125,000–250,000
2	Aerosporin	Polymixin A (a polypeptide)		Spleen	>10,000	310–625
				Skin	800–1600	200–400
3	Alcohol	100% ethanol (solvent & antiseptic)	46.07	Spleen	>250,000	3,500–5,000
				Skin	>80,000	2,000–4,000
4	Antistine	2-(N-benzyl-anilino-methyl) imidazoline HCL antihistaminic	301.80	Spleen	500–1000	1.25–2.5
				Skin	160–400	15–40
5	Ascorbic acid	Vitamin C	176.12	Heart	250- 2,500	25–250
6	Asterol dihydrochloride	6-(B-diethyl amino ethoxy)-2-dimethyl amino-benzothiazole Dihydrochloride	366.35	Skin	200–800	20–40
				Cord	625–1,250	5–10
				Heart	625–1,250	5–10
				Spleen	156–312	10–20
7	Atropine HCL	(Alkaloidal convulsant)	325.86	Cord	40	4
8	Aureomycin HCL and Na glycinate	(Antibiotic)		Spleen	>5,000	150–300
				Skin	830–1250	100–200
9	Bacitracin	(Antibiotic polypeptide)		Spleen	>20,000	625–1250
				Skin	5,000–10,000	800–1600
10	Barbituric acid, Na salt	Allyl-1-methyl-allyl-(hypnotic)	254	Cord	1,250	
				Heart	1,250	
11	Barbituric acid, Na salt	sec-butyl-2-methyl-allyl-(hypnotic)	270	Cord	1,250	
				Heart	1,250	
12	Barbituric acid, Na salt	1,3-dimethylbutyl-allyl (convulsant)	284	Cord	156	
				Heart	156	
13	Barbituric acid, Na salt	1,3-dimethyl butyl-ethyl (convulsant)	272	Cord	312	
				Heart	1,250	
14	Barbituric acid, Na salt	3,3-dimethyl-ethyl-allyl-thio- (convulsant)	263	Cord	62	
				Heart	1,250	
15	Barbituric acid, Na salt	3,3-dimethyl-diallyl-thio-(convulsant)	268	Cord	312	
				Heart	1,250	
16	Barbituric acid, Na salt	3,3-dimethyl-diallyl-thio-(convulsant)	284	Cord	78	
				Heart	156	

No.	Substance	Identity	M.W.	Test tissue	Concentration in γ/ml. of medium	
					Outgrowth stops	Detectable injury
17	Barbituric acid, Na salt	3,3-dimethyl-ethyl-allyl- (convulsant)	256	Cord	1,250	
				Heart	1,250	
18	Barbituric acid, Na salt	Ethyl-3-methyl allyl- (hypnotic)	242	Cord	1,250	
				Heart	1,250	
19	Barbituric acid, Na salt	Isobutyl-1-methyl-allyl-thio- (convulsant)	277	Cord	1,250	
				Heart	1,250	
20	Barbituric acid, Na salt	Isopropyl-3-methyl-allyl- (hypnotic)	256	Cord	1,250	
				Heart	1,250	
21	Barbituric acid, Na salt	N-methyl-3,3-dimethyl-ethyl-allyl-(convulsant)	261	Cord	62	
				Heart	1,250	
22	Barbituric acid, Na salt	N-propyl-1-methyl-allyl (hypnotic)	256	Cord	1,250	
				Heart	1,250	
23	Barbituric acid, Na salt	N-propyl-2-methyl-allyl (hypnotic)	256	Cord	1,250	
				Heart	1,250	
24	Benadryl	2-(Benzhydryloxy)-N,N-dimethyl ethylamine HCL (antihistaminic)	328.26	Spleen	250–500	1–2.5
				Skin	165–330	30–40
25	Biotin	cis-Hexahydro-2-040-1H-thieno(3,4)-imidazole-4-valeric acid; (vitamin H)	244.31	Cord	>180	37–92
26	Benzedrine	1-phenyl-2-amino propane; amphetamine (vasopressor)	135.2	Cord	500	80
27	Boric acid	H_3BO_3 (antiseptic)	61.84	Cord	156–312	20–40
				Heart	312–625	40–80
				Spleen	>620	80–160
28	Caffeine	1,3,7-trimethyl xanthine (stimulant)	194.19	Cord	5000	625
29	Carbitol	Diethylene glycol mono-ethyl ether (solvent)	134.17	Spleen	25,000–50,000	1,250–2500
				Skin	16,000–40,000	830–1660
30	Carbowax 1500 W	Soft mixture of poly-ethylene glycols (solvent)	500–600	Spleen	>250,000	12,500–25,000
				Skin	20,000–40,000	2,000–4,000

31	Carbowax 1540	Soft mixture of polyethylene glycols (solvent)	1300-1600	Spleen Skin	125,000–250,000 >40,000	1,250–2,500 4,165–8,330
32	Carbowax 4000	Hard mixture of polyethylene glycols (solvent)	3000–3700	Spleen Skin	125,000–250,000 20,830–40,000	2,500–6,250 4,165–8,330
33	Cellusolve	2-Ethoxyethanol (solvent)	90.12	Spleen Skin	25,000–50,000 16,660–41,660	1,250–2,500 830–1,660
34	Chloral hydrate	2,2,2-trichloro-1,1-ethanediol (Hypnotic)	165.42	Cord	750	30
35	Chloramycetin	Chloramphenicol; D-threo-2-dichloro-acetamido-1-p-nitrophenyl-1,3-propanediol (antibiotic)	323.14	Spleen Skin	1665–3330 555–830	165–330 135–275
36	Cholesterol	(metabolite)	386.64	Cord Heart Spleen	75–150 75–150 75–150	18–36 9–18
37	Circulin	(Polypeptide antibiotic)		Spleen Skin	>20,000 3,000–5,000	2,500–5,000 415–830
38	Cocaine	Benzoyl-methyl ecgonine (convulsant)	303.35	Cord Spleen Skin	400 2,500–5,000 1,665–3,330	50 30–60 205–410
39	Coramine	Pyridine-3-carloxylic acid diethylamide (convulsant)	178.23	Cord	6,250	2,100
40	Cortisone acetate	17-hydroxy-11-dehydrocorticosterone acetate (adrenal cortex hormone)	402.24	Cord Spleen Heart	75–150 75–150 75–150	4.7–9.4 4.7–9.4 9.4–18.8
41	Croton oil	Fixed oil (vesicant)		Heart Spleen	5,125–10,250 5,125–10,250	
42	Di-isopropyl fluorophosphate	(Esterase inhibitor)	208.17	Cord Heart Spleen	312–3,125 3,125–6,250 6,250–12,500	15–156 156–312 7.8–15.6
43	Dromoran	3-Hydroxy-N-methyl morphinan (narcotic)	257.36	Cord Heart Spleen	250–500 500–1,000 500–1,000	4–8 62–125 15–30
44	Endomycin	(Polypeptide antibiotic)		Spleen Skin	2,500–5,000 250–500	100–200 50–100

No.	Substance	Identity	M.W.	Test tissue	Concentration in γ/ml. of medium	
					Outgrowth stops	Detectable injury
45	Ephedrine HCL	(Vasopressor)	201.7	Cord	830	
46	Epinephrine HCL	3,4-dihydroxy-a-methyl-amino ethyl-benzyl alcohol (vasopressor)	219.6	Cord	250	30
47	Erythromycin	(Polypeptide antibiotic)		Skin	345–690	85–170
				Spleen	520–1,040	30–60
48	Flaxedil	1,2,3-tris (triethyl ammonium ethoxy) benzene tri iodide (muscular relaxant)	891.56	Cord	625–1,250	1.25–2.5
				Heart	5,000–10,000	2.5–5.0
				Spleen	5,000–10,000	5–50
49	Furacin	5-Nitro-2-furaldehyde semicarbazone (bacteriostatic)	198.14	Spleen	>100	12–25
				Skin	8–16	0.5–1
50	Gantrisin	N-(3,4-Dimethyl-5-isoxazolyl) sulfanilamide (bacteriostatic)	267.3	Skin	1,665–3,330	205–410
51	Glutamic acid	2-amino pentanedioic acid (metabolite)	147.1	Cord	750	6
52	Gramacidin D	(antibiotic)		Spleen	>500	5–10
				Skin	0.5–1.0	0.1–0.5
53	Histadyl	2-(2-Dimethylamino ethyl)-2-thenylaminol pyridine (antihistaminic	297.78	Spleen	625–1250	12–25
				Skin	210–420	25–50
54	Histamine dihydrochloride	2-(4-imidazolyl) ethylamine dihydrochloride (cellular irritant)	184.08	Cord	625–1250	39–78
				Heart	625–1250	313–625
				Skin	2,080–4160	415–830
55	Intocostrin	Standardized curare extract (muscle relaxant)		Heart	5–10	2
				Spleen	>10	
56	Lobelline hydrochloride	(Alkaloidal respiratory stimulant)	373.8	Cord	100	50
57	Mercuric chloride	$HgCl_2$ (antiseptic)	271.52	Cord	6.3–12.6	0.4–0.8
				Heart	6.3–12.6	0.8–1.6
				Spleen	6	3.1–6.2

58	Merthiolate	Na ethylmercuri thiosalicylate (antiseptic)	404.8	Spleen	25–50	0.125–0.25
				Skin	8–16	0.8–1.6
59	Methylparaben	Methyl-p-hydroxy benzoate (preservative)	152.14	Spleen	520–1040	30–60
				Skin	175–350	140–175
60	Metrazol	6,7,8,9-tetrahydro-5-azepotetrazole (convulsant)	138.17	Cord	12,500	7,500
61	Neoantergan	2-(2-dimethyl-amino-ethyl)p-methoxy benzyl) aminopyridine HCl (antihistaminic)	321.78	Cord	125–250	8–16
62	Neomycin sulfate	(Antibiotic complex)		Spleen	250,000–500,000	780–1,560
				Skin	4,160–6,250	830–1,010
63	Nicotine	1-Methyl-2-(3-pyridyl) pyrrolidine (convulsant)	162.23	Cord	830	250
64	Nicotinamide	3-pyridine carboxylic acid amide (vitamin)	122.12	Cord	25,000	3,000
65	Panthenol	N-Pantoyl-3-propanolamine (B complex vitamin)	205.25	Cord	5,000–10,000	313–625
				Heart	5,000–10,000	<1000
				Spleen	5,000–10,000	625–1,250
66	Para-aminobenzoic acid	(metabolite)	137.13	Cord	no toxicity at saturation	
67	Paraldehyde	para acetaldehyde (hypnotic)	132.16	Cord	2,500	250
68	Paredrine	dl-p-hydroxy-methyl-phenethyl amine (vasopressor)	151.2	Spleen	1,250–2,500	12.5–25
				Skin	830–1,660	105–210
69	Penicillin G (potassium)	potassium benzyl penicillinate (antibiotic)	372.47	Skin	2,000–3,750	1,500–1,875
70	Penicillin G (sodium)	sodium benzyl penicillinate (antibiotic)	356.38	Spleen	75,000–150,000	940–1,875
				Skin	5,000–10,000	1,250–2,500
71	Penicillin O	Allylmercapto ethyl penicillin (antibiotic)		Spleen	150,000–300,000	1,875–3,750
				Skin	2,500–5,000	625–1,250
72	Pentobarbital sodium	Na-5-ethyl-5-(1-methyl butyl) barbiturate (Hypnotic)	248.26	Cord	156–312	
				Heart	156–312	
73	Perazil	1-(4-Chlorobenzhydryl)-4-methyl piperozine dihydrochloride (antihistaminic)	373.75	Spleen	1,250–2,500	6–12
				Skin	100–165	4–8

No.	Substance	Identity	M.W.	Test tissue	Concentration in γ/ml. of medium	
					Outgrowth stops	Detectable injury
74	Phenol	Hydroxybenzene (antiseptic)	95.12	Spleen	1000–2000	10–20
				Skin	830–1660	100–200
75	Phenargen	10-(2-dimethylamino-1-propyl) phenthiazine (antihistaminic)	284.4	Cord	6.2–62	0.8–1.6
76	Phenyl mercuric acetate	(Antiseptic)	336.75	Heart	10–20	2.5–5
77	Picrotoxin	$C_{30}H_{34}O_{13}$, (convulsant)	602.37	Cord	>3,750	500
78	Polymyxin B sulfate	(Polypeptide antibiotic)		Spleen	>10,000	310–620
				Skin	830–1,660	105–210
79	Presidon	3,3-diethyl-2,4-dioxo-tetrahydro pyridine (hypnotic)	169.2	Cord	3,750–5,080	187–376
				Heart	3,750–5,080	750–1,500
				Spleen	3,750–5,080	375–750
80	Procaine HCL	2-diethyl amino-ethyl-p-amino benzoate HCl (local anesthetic)	272.7	Cord	50–100	12.5–25
				Heart	25–50	3–6
				Spleen	625–1,250	1.6–3.2
81	Progesterone	Pregnene-3,20-dione (corpus luteum hormone)	314.45	Cord	75–150	<10
				Heart	75–150	<10
				Spleen	37.5–75	<10
82	Proprionic acid	CH_3CH_2COOH (fungicide)	74.08	Cord	6.2–12.5	0.39–0.78
				Heart	3.1–6.2	0.39–0.78
				Spleen	12.5–25	0.08–0.16
83	Propylene glycol	1,2-Propanediol (solvent and fungicide)	76.09	Spleen	62,500–125,000	2,500–5,000
				Skin	10,400–20,800	83,330–166,660
84	Prostigmine Br	(m-Hydroxyphenyl) trimethyl ammonium bromide trimethyl carbonate (parasympathomimetic)	303.2	Cord	125–250	16–32
				Heart	250–500	31–62
				Spleen	250–500	16–32
85	Pyribenzamine HCl	2-(Benzyl (2-dimethyl aminoethyl) amino) pyridine HCl (antihistaminic)	291.8	Spleen	500–1,000	2.5–5
				Skin	210–420	20–40
86	Pyromen	(polysaccharide antasthmatic)		Cord	125,000–250,000	7,810–15,620
				Heart	62,500–125,000	3,900–7,800
				Spleen	125,000–250,000	7,810–15,620

87	Quotane	1-(B-dimethyl amino ethoxy)-3-butyl isoquinoline HCl (anti-irritant)		Spleen	625–1,250	10–20
				Skin	125–250	2–3
88	Resorcinol	$C_6H_6O_2$ dihydroxybenzene (anti-irritant)	110.11	Cord	625–1,250	78–156
				Heart	250–1,250	312–625
				Spleen	250–1,250	156–312
				Skin	500–1,000	2–3
89	Rhus	Poison ivy extract (irritant)		Cord	75–150	9.4–18.8
				Heart	75–150	18.8–37.5
				Spleen	>150	9.4–18.8
90	Ricinoleic acid	$C_{18}H_{34}O_3$ (irritant)	298.45	Heart	10,000–20,000	2,500–5,000
91	Salicylic acid	Hydroxybenzoic acid (keratolytic)	138.12	Spleen	>1,000	25–50
				Skin	>166	40–80
92	Seconal sodium	Na5-allyl-5-(1-methylbutyl) barbiturate (hypnotic)	260.27	Cord	156–1,250	
				Heart	156–312	
93	Scopolamine HB	Hyoscine (hypnotic)	438.32	Cord	1,250–2,500	780–1,560
94	Sodium amytal	Na 5-ethyl-5-isoamyl barbiturate (hypnotic)	248.26	Heart	156–312	78–156
95	Sodium bromide	Na Br (hypnotic)	102.9	Cord	20,000	6,250
96	Sodium chloride	NaCl (metabolite)	58.45	Cord	6,250–12,500	1,560–3,120
				Heart	6,250–12,500	1,560–3,120
				Spleen	>12,500 (above media content)	196–392 (above media content)
97	Sodium proprinate	CH_3CH_2COONa (fungicide)	96.07	Cord	>5,000	156–312
				Heart	2,500–5,000	78–156
				Spleen	>2,500	125–250
98	Streptomycin	C_2, $H_{39}N_7O_{12}$ (antibiotic)	581.58	Spleen	>300,000	155–310
				Skin	2,220–6,660	210–415
99	Strychine sulfate	(Alkaloidal convulsant)	856.96	Cord	16	6
100	Surfacaine	Cyclomethycaine HCl (local anesthetic)	395.96	Spleen	750–1,500	30–60
				Skin	415–830	50–100
101	Syncurine	Decamethonium bromide (muscle relaxant)	490.37	Cord	500–1,000	62–124
				Heart	500–1,000	62–124
				Spleen	1,000–5,000	125–250
102	Synkavite	menadione tetrasodium diphosphate (synthetic vitamin K)	530.2	Cord	20–25	6.2–12.5
				Heart	25–30	16–20
				Spleen	60–120	3–6

No.	Substance	Identity	M.W.	Test tissue	Concentration in γ/ml. of medium	
					Outgrowth stops	Detectable injury
103	Tensilon	(3, Hydroxyphenol) Dimethylammonium bromide (muscle relaxant)	246.15	Cord	250–2,500	62.5–125
				Heart	2,500–5,000	156–312
				Spleen	>5,000	625–1,250
104	Terramycin HCl	(Antibiotic)	496	Skin	100–200	25–50
105	Thephorin	2-Methyl-9-phenyl-2,3,4,9-tetrahydro-1-pyridindene (antihistaminic)	261.35	Cord	156–250	124
				Heart	250	62–124
				Spleen	1,000–2,000	62–124
				Skin	25–30	2–2.5
106	D-Tubocurarine chloride	(Alkaloidal muscle relaxant)	695.66	Cord	750–1,500	375–750
				Heart	>1,500	750–1,500
				Spleen	>1,500	180–360
108	Tween	("Span" type solvent)		Cord	5,320–10,640	105–210
				Heart	5,320–10,640	210–420
				Spleen	5,320–10,640	210–420
108	Tween 80	(Polysorbate solvent)		Cord	100,000–250,000	1,562–3,125
				Heart	100,000–250,000	3,125–6,250
				Spleen	>250,000	3,125–6,250
109	Undecylenic acid	$C_{11}H_{20}O_2$ (fungicide)	184.27	Cord	80–159	20–40
				Heart	159–318	40–80
110	Zinc undecylenate	(fungicide)	433.82	Cord	62.5–125	0.25–0.5
				Heart	62.5–125	1–2

All observations were made on primary explants, which have the disadvantage that many cells are dying and that their products in various degrees may bind the drug under study. However, this procedure is accessible to a larger number of workers, and it is simpler, cheaper, and quicker than tests performed with pure cell strains.

Obviously, a comprehensive study of drug toxicity should include tissues whose vulnerability to injury may constitute the immediate cause of death of the organism. It would accordingly be ideal to have data based on the reaction of brain, liver, and kidney tissues of human origin in the description of pharmaceutically useful chemicals. Here again our data represents expediency.

Because of their availability and ease of management, chick embryonic tissues are generally used for the first step in screening operations. When warranted, a drug whose toxicity has been easily evaluated with chick explants may be studied with the more troublesome procedures utilizing human tissue explants. Since we have had excellent collaboration from dermatologists and plastic surgeons, our tables of data are enriched at several points with toxicity evaluations based on adult human epidermal cells.

As end points, effort has been directed at determining (1) the minimal quantity or concentration required for the total inhibition of outgrowth, the "minimal inhibitor dose" (MID) or the concentration at which growth stops; and (2) the value representing that quantity or concentration of the chemical which produces the slightest demonstrable effect, the "least injurious dose" (LID). Since biological measurements inherently defy absolute precision of measurement, it has been found expedient to report four values. The mean MID therefore lies somewhere between the concentration which produces complete inhibition and that which permits the emigration of a few cells. Similarly, the mean LID lies between values permitting outgrowth identical with untreated controls and cultures showing recognizable minimal injury to the system of emigrating cells.

Further definition of graded injury has been reported from this laboratory with the use of rating schemes based upon the distance traversed by emigrating cells or of nerve fibers. In practice, such measurements are made by placing the explant at one edge of the optical field and rating outgrowth as ±, +, ++, +++, and ++++, with the highest value representing emigration to the opposite margin of the microscope field. Whenever such ratings are made, it is necessary to define the system of optics which is employed. For the present study, graded responses evaluated in this manner are not reported. A more detailed description of the methods for short term toxicity studies has been given by Pomerat (1951).

Information which has accumulated to date is presented for convenience in tables arranged in alphabetical order. The molecular weight of the compounds is given where precise data is available. In the columns entitled "tissues," the indication "spleen" refers to fragments from 18- to 20-day chick embryos; "spinal cord" to fragments from the 9th-day chick embryo, while explants from heart and intestines were obtained from 9- to 12-day chick embryos. "Skin" refers to epithelial emigration from explants of adult human sources, usually obtained in the course of skin-grafting procedures. Toxicity values are given in γ/ml. of culture medium.

It is hoped that work in progress as well as aid from investigators in other laboratories may lead to the enrichment of the framework set up in these provisional tables. In addition to the value of such information for its own sake, it is obvious that, for studies designed to introduce chemicals in perfusion chambers containing cells whose activities are being recorded with cinematographic techniques, it is essential to have some knowledge of the concentration levels which may prove useful in achieving the goals of the experiment.

It is not pertinent to discuss detailed aspects of the summarized findings which are reported here. Thorough consideration has already been given, with full analysis of related reports by Ivers, Pomerat, and Niedhardt for various hormones; by Painter, Pomerat, and Ezell for certain drugs influencing the nervous system; by Hudspeth, Swann, and Pomerat for oxygen; by Everett, Pomerat, Hu, and Livingood for dermatological drugs; by Frantz and Pomerat for "presidon"; by Hu, Holmes, Pomerat, Livingood, and McConnell for P^{32}; by Ruskin, Pomerat, and Ruskin for cortisone; by Everett, Livingood, Hu, and Pomerat for contact allergens; by Hu, Livingood, Johnson, and Pomerat for antibiotics; and by McCormick and Knicker for morphine, "dromoran," and amytal in regard to addiction tolerance. A brief review will be offered here, summarizing some of the more significant findings.

The high toxicity for skin growth shown by various antihistaminics is interesting in relation to clinical experience. The topical use of anti-infective compounds has been under criticism for some time, as in the case of penicillin or sulfa ointments. These compounds have been attacked as causing excessive skin "sensitivity," enough to outweigh their therapeutic value in skin infections. Topical preparations of antihistaminics have been condemned for the same reason. The Council on Pharmacy and Chemistry of the American Medical Association has recently removed such topical antihistaminic preparations from acceptance in *New and Non-Official Remedies* on the basis of evidence of "producing cutaneous sensitivity." The dermatitis produced by such preparations may be caused by the factors responsible for the relatively high toxicity of antihistaminics for skin growth as revealed by tissue-culture methods. At any rate, such methods provide a practical way of screening antihistaminics with regard to possible skin injury from topical application.

On similar evidence, the high toxicity for skin growth of such anti-irritant dermatologic compounds as "quotane" and resorcinol might indicate potential danger of dermatitis from too prolonged application. The toxic concentrations of various antibiotics, as reported by Hu and her associates, for skin growth in tissue culture, also give indications of danger in topical application from gramicidin and from terramycin. It yields, however, no suggestion of the possibility of "sensitivity" reactions in the skin to the topical application of penicillin.

All alkaloids tested show relatively strong injurious effect on tissue growth, with the exception of caffeine, scopolamine, and tubocurarine. It is surprising that procaine should be more inhibitory to growth than cocaine. The high toxicity of alkaloids to tissue culture growth may reflect a specific antimetabolic effect.

In considering ordinary metabolites, however, it is amazing to note the relatively strong blocking of growth of such presumed essential metabolic agents as ascorbic acid, biotin, cholesterol, glutamic acid, and even sodium chloride (for spleen). Cortisone, which was carefully studied by Ruskin and her associates, also appears to be rather toxic to growth of representative tissues *in vitro*. It may be that these metabolites require optimum concentrations in relation to many other compounds in the huge complex of metabolism in order to function smoothly. Excesses may block the essential processes at some decisive point. On the other hand, other essential metabolites, as folic acid, nicotinamide, panthenol, p-aminobenzoic acid, and menadione are tolerated in relatively strong concentrations without interfering with growth. Perhaps these are "essential" for growth rather than for some specific functional activity.

As would be expected, protein precipitating antiseptic agents, as mercuric chloride, merthiolate, phenylmercuric acetate, and zinc undecylinate, powerfully interfere with growth. The observed action in tissue culture of phenol, boric acid, and of salicylic acid seems to be in line with their status relative to heavy metal antiseptics as derived from other data. However, the high tolerance of tissue cultures to acetone is surprising. Alcohol causes injury at concentrations approaching the range obtained by usual methods. The glycol solvents are relatively nontoxic to tissue culture, as would be anticipated from general data about them. The compound most injurious in tissue culture was found to be the fungicide, proprionic acid.

Interesting are the data on the tissue-culture toxicity of compounds acting on the nervous system. These data have been skillfully analyzed by Painter and his associates in relation to toxicity figures obtained by conventional methods on whole animals. Here it is clearly shown that the entire mammal is more sensitive to these kinds of compounds than is an "isolated" nerve-cell system, in equivalent concentrations. It is remarkable that five of eight barbital convulsants should show much higher toxicity on spinal cord growth than any of the barbital hypnotics. In general, the alkaloidal nervous depressants exhibit greater toxicity for tissue cultures than the aliphatic hypnotics and sodium bromide. Similarly alkaloidal nervous system stimulants seem to be more injurious to spinal cord explants than are depressants. On the other hand, such convulsant drugs as metrazol and coramine are relatively nontoxic. Muscle-relaxing agents are rather remarkably nontoxic to tissue cultures.

The interesting observation was made by McCormick and Knicker that lung, skin, and omentum from addicted animals is more tolerant in tissue culture to morphine and "dromoran" than is the case with these tissues from nonaddicted animals. In sodium amytal addiction, however, these tissues were found to be more sensitive than the nonaddicted controls.

Summary

A discussion has been offered of some of the possible ways by which tissue culture methods may be used in pharmacological investigations. Advantages and disadvantages have been considered. Screening of compounds for injury to various tissues with particular reference to growth is a relatively simple and helpful procedure. Chemotherapeutic screening also has promise. Drugs for certain specific action, as well as tissue metabolites and antimetabolites may be conveniently examined by tissue culture techniques. Important is the dynamic aspect of tissue and cell response to drugs which may be well recorded by time-lapse cinematography using phase-contrast microscopy.

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Discussion of the Paper

Doctor Marvin A. Schneiderman (*National Cancer Institute, National Institutes of Health, Bethesda, Md.*): The table of Pomerat and Leake giving, effectively, a pair of MID (minimum inhibitory dose) values and a pair of LID (least injurious dose) values is a useful step away from the concept of "critical ratio" or "therapeutic index" as a measure of the useful potential of a drug.

Just as Trevan,[1] in 1927, introduced into pharmacology the notion of a distribution, among test animals, of tolerances to a drug, there now similarly appears to be a need to consider the different distributions of responses to a drug when the drug is capable of inducing conflicting responses. Probably every material, if given in sufficiently large quantities, will produce damaging effects. Thus every drug can be considered to have two effects, one positive and one negative, which must be weighed against each other. The first effect is the desired effect, the "cure" of cases of illness or, in the context of tissue culture (and cancer), perhaps we could say "damage to cancer cells." The undesired effects, in animal experimentation (and, of course, in the administration of drugs to humans) are "drug deaths," *i.e.*, death resulting from the effects of the drug. In tissue culture in the cancer field, this result would be equivalent to damage to "normal" cells or to "normal" tissue.

A first attempt at evaluating these contrary effects is given by the rule-of-thumb "critical ratio," which is the ratio of that dose which just begins to have a deleterious effect, to the smallest dose which has some desired effect. In the usual language, this is the MTD/MED, maximum tolerated dose divided by minimum effective dose. These concepts are deficient in the same sense that the deficiencies in the concept ". . . there is a dose, for any given poison, which is only just sufficient to kill all or most of the animals of a given species, and that doses very little smaller would not kill any animals of that species" led Trevan into the dose-response relationships, and to the ED_{50} ideas now so extensively used.

The two numbers for MID and the two numbers for LID give the basis for dose-response curves, and thus the initial impetus for moving away from the "critical ratio."

In the charts that follow, the ideas of Pomerat and Leake are extended, and an alternative to the critical ratio-therapeutic index concept is proposed. These ideas have been used by Golden and his associates in the study of anti-leukemic drugs in mice.[2]

In FIGURES 1 to 6, the usual sigmoid dose-response curves have been transformed to straight lines, as in "Probit analysis." The choice of transformation is of no consequence here, probits having been chosen because of the general knowledge of the techniques among pharmacologists. There is one additional convention adhered to in these figures. The desired effect response curve ("damage to cancer cells") is drawn as the same line in all the figures. The undesirable effect curve ("damage to normal cells") is shifted in each of the figures in order to bring out the desired points.

Why is the "critical ratio" an inadequate measure? FIGURES 1, 2, and 3 are all drawn so that the critical ratio is 4:1. It is assumed that the 2 per cent level* is sufficiently low so that we can call the dose which damages 2 per cent of the cancer cells as MED, and the dose which damages 2 per cent of the normal cells as MTD. However, FIGURES 1, 2, and 3 represent substantially different situations.

Consider a drug in terms of its allowable "cost." Define this allowable

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* The concepts of MED and MTD are sufficiently imprecise so that any small percentage could have been chosen. The 2 per cent figure is used here only for convenience. If some other percentage were chosen, it might have been necessary to redraw FIGURES 1 to 6, but the conclusions would have been the same.

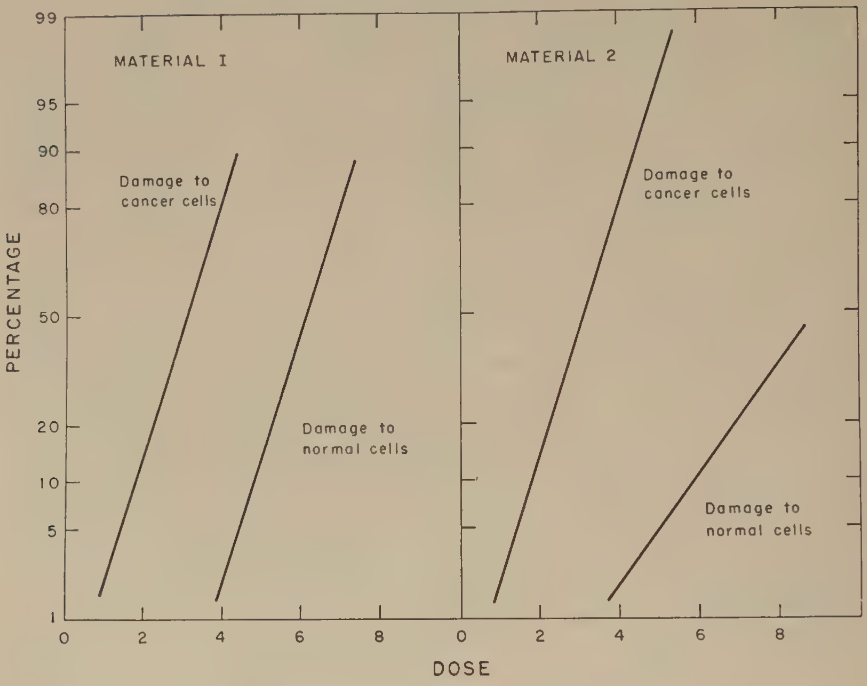

FIGURE 1

FIGURE 2

cost as (for example) 5 per cent damage* to normal cells. It is worth asking, then, "What per cent desired effect do we get for 5 per cent undesired effect?" In FIGURE 1, there is 89 per cent damage to cancer cells for 5 per cent damage to the normal cells. In FIGURE 2, a material with the same "critical ratio," there is over 96 per cent undesirable effect for 5 per cent undesirable effect. In FIGURE 3, again with a critical ratio of 4:1, for a 5 per cent damage "cost" the material gives 83 per cent desirable results.

FIGURES 4 and 5 represent materials with a critical ratio of 3:1. For 5 per cent "cost," material 4 gives 99.9 per cent desirable effect, obviously a very good material. Material 5, in FIGURE 5 is poorer. A damage cost of 5 per cent gives only 57 per cent desired effects.

FIGURE 6 is a pathological case. Here the critical ratio is 1. This is the sort of material that probably would never be used. For a 5 per cent "cost," however, there is an 80 per cent desirable effect—possibly better than the material in FIGURE 5, with a 3:1 ratio.

What is proposed here is thus an obvious extension of the Pomerat-Leake concepts. Rather than use the critical ratio, this procedure is suggested:

(a) From substantive considerations, such as the importance or seriousness of the condition against which the drug is effective, an allowable "cost" in

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* This, too, is an arbitrary number. The choice of the "cost" level is of utmost importance, and is discussed below. Since it would be possible to shift the order of preference of materials by shifting the "cost" level when different materials are compared, they must be compared for the same "cost."

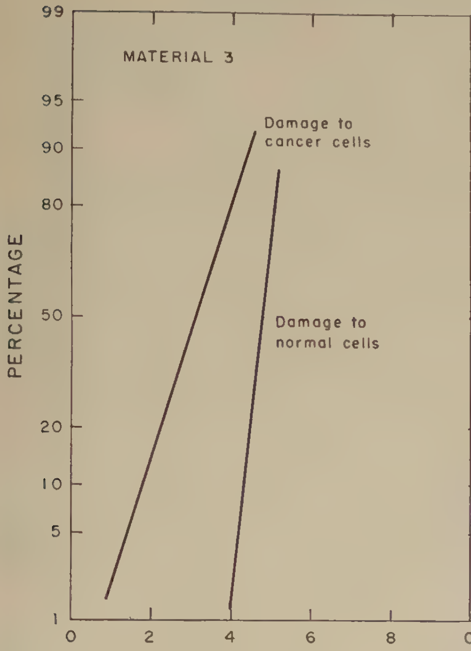

FIGURE 3

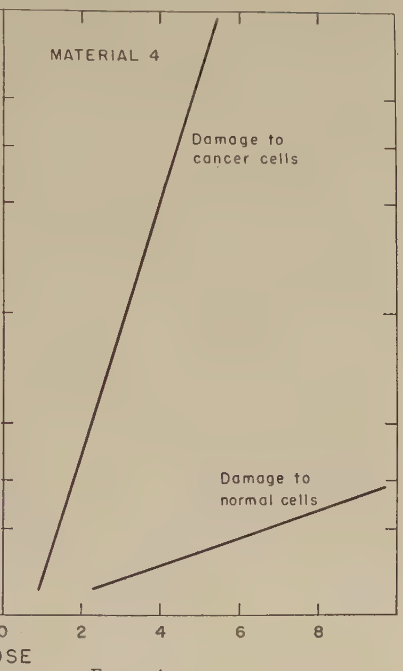

FIGURE 4

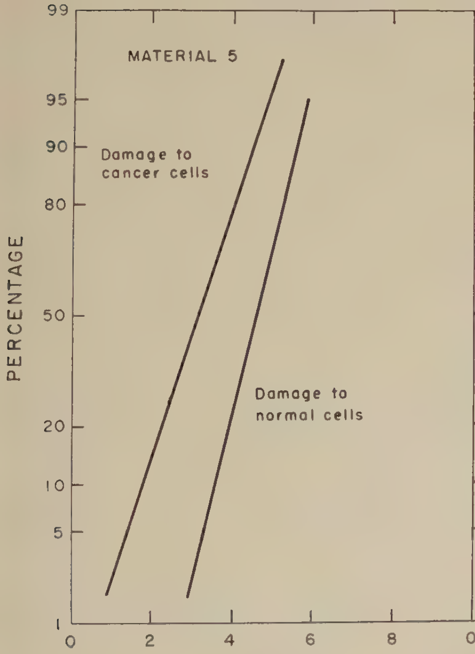

FIGURE 5

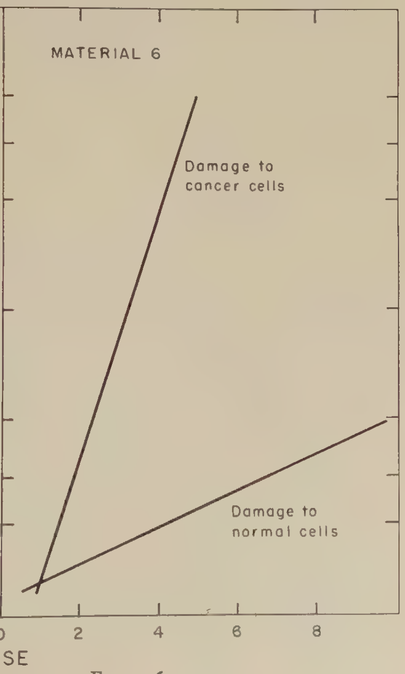

FIGURE 6

damaging effects is determined. If, accordingly, we were considering an anticancer drug for use with humans afflicted with incurable cancer we might be willing to permit a fairly high "cost" in drug deaths. If we were considering a headache remedy, we might be willing to permit only a very low "cost" in bad side effects.

(b) Determining this "cost" also determines the dose at which this cost occurs. The per cent cures (or "damage to cancer cells" in FIGURES 1 to 6, given here) is then determined for this dose.

(c) By comparing the per cent "cures" for different materials, it is now possible to select for use those materials which have the highest per cent cures for the given cost.

(d) Where two or more materials give the same per cent "cures" for a given cost, the "critical ratio" can be used to select among these materials. The material of choice among those equal under the "cost" technique would be the one with the highest critical ratio.

Obviously, the key to this procedure is the proper selection of an allowable "cost." For some illnesses or experimental studies, the only allowable cost would be a cost of little more than zero. For extremely serious illnesses, where cures at almost any cost are being sought, a cost figure as high as 5 per cent "drug deaths" may be allowed.

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ASSAY OF CARCINOLYTIC AND CARCINOSTATIC AGENTS*

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Our use of tissue cultures in the assay of chemicals for possible selective toxicity to neoplastic mammalian cells is based on that of Cornman (1944a, b), who discovered a selectively damaging effect of yellow penicillin on rat sarcoma cells *in vitro*, and on the procedures used by Ormsbee and Cornman (1948). With their methods, they were able to demonstrate a preferential damage to mouse sarcoma cells by nitrogen mustard (Karnofsky *et al.*, 1947; Cornman and Ormsbee, 1947) and by podophyllin (Ormsbee, Cornman, and Berger, 1947).

It was recognized by Ormsbee and his co-workers (Karnofsky *et al.*, 1947) that a given agent might have certain effects in tissue culture that are not observed in the animal. However, part of the reason for a tissue-culture assay lies in the possibility of making a test when only minute quantities of a given agent, too small for trial in animals, are available. It accordingly becomes of value to determine how reliable a prediction of effect in animals may be made, based on results in tissue culture. Limitations of our assay technique with respect to such predictions will become evident in the following account of results obtained with several series of compounds. These compounds included agents definitely inhibitory to growth of sarcoma-180 in mice, related compounds of slight inhibitory power, and ineffective analogs.

Procedure

Our assay is simply a comparison of the toxicities of a given agent to embryonic and sarcomatous or carcinomatous cells of the mouse. Conceivably, such toxicity comparisons could be carried out in a number of ways, more or less of equal validity *a priori*. Our procedure, which has been described elsewhere (Biesele, 1951; Biesele *et al.*, 1951), may be summarized as follows:

Six fragments of healthy mouse sarcoma-180 tissue, each about 1 or 2 millimeters in diameter, are planted in a row in the bottom third of a roller tube under a chicken-plasma clot. A similar row of six fragments of normal mouse tissue is planted opposite in the tube. The normal tissue is usually abdominal skin of Ak embryonic mice about two thirds through the gestational period. Sometimes newborn mouse hearts have served as the source of normal cells.

One milliliter of a supernatant medium of the following composition is added to each tube: Gey's balanced salt solution, four parts; 50 per cent chick-embryo extract in Gey's solution, two parts; human placental-cord serum, one part; and horse serum, three parts. Each milliliter of medium contains 25 units of penicillin G and 25 micrograms of streptomycin.

The tubes are then incubated at 37° C. in a rotor revolving 10 times per hour.

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After incubation overnight, the cultures are examined under low power of the microscope, and those showing poor growth or contamination are discarded.

The cultures are then dosed with a series of concentrations of the agents to be tested. The agents are dissolved or suspended in a suitable innocuous vehicle, ordinarily physiological saline. One tenth milliliter of the culture medium is removed and replaced with an equal volume of the dosing solution or suspension. Duplicate tubes are used with each concentration of agent. Several control tubes are treated with the vehicle alone.

After another 24 hours of incubation, each culture is again examined under low power of the microscope. Damage to the cells is assessed on the basis of rounding up, granularity, disintegration, and the inhibition of outgrowth beyond that attained before administration of the agent.

If there is no differential in damage to normal and neoplastic cells, and if no gradient of effect over the concentration series is noted with cells of one tissue or the other, ordinarily a second trial with higher or lower concentrations is desirable.

In this manner, a statement can be made concerning the relative toxicity of the agent to cells of the particular normal mouse tissue and the particular mouse neoplasm in tissue culture under the conditions employed.

A more elaborate assessment of cellular damage can be made by examination with oil-immersion phase-contrast microscopy. For this purpose, we have planted cultures on coverslips inserted into the roller tubes and applied to flattened regions of the tube wall with a plasma clot. For examination, the coverslip is removed from the culture vessel, the clot on the back is gently wiped off, and the coverslip is placed face down in a drop of the culture medium on an ordinary glass microscope slide. The cultures are then rapidly scanned, and representative cells are photographed in the first few minutes.

Additional information has been obtained with some agents by determining the proportions of mitotic nuclei and of pyknotic nuclei in the outgrowth. Again, cultures grown on coverslip inserts in flattened roller tubes are used. These cultures are fixed in alcohol-acetic acid 3:1 for 30 minutes, stained with the Feulgen nucleal reaction, and counterstained with light green. Counts of 1000 nuclei in the outgrowth are made in a band or bands running from the outgrowth edge tangentially past the edge of the original explant. Nuclei in each phase of mitosis are enumerated, as are pyknotic nuclei, and a record is kept of aberrant mitoses. Such counts are made on several sarcoma cultures and several embryonic tissue cultures for each concentration of agent.

Results

Effects observed with differentially toxic purines are illustrated in FIGURES 1 to 4, which are phase-contrast photomicrographs of control cells and of others treated for six hours with 0.1 millimolar 2,6-diaminopurine lactate.* Sarcoma-180 cells under treatment withdraw their processes and become more granular, their nuclei become pyknotic and, after a day, many of the cells have disintegrated. At this concentration, cells of treated embryonic mouse skin do

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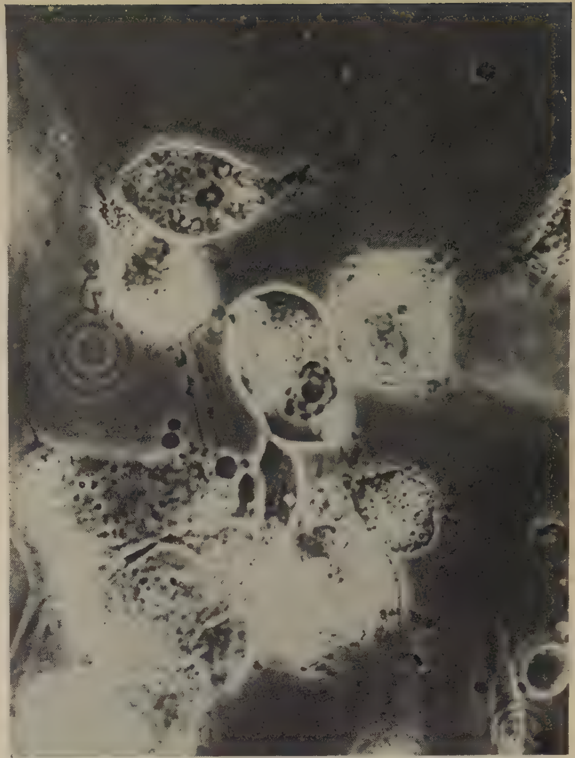
* Kindly supplied by Doctor George H. Hitchings of the Wellcome Research Laboratories, Tuckahoe, N. Y.



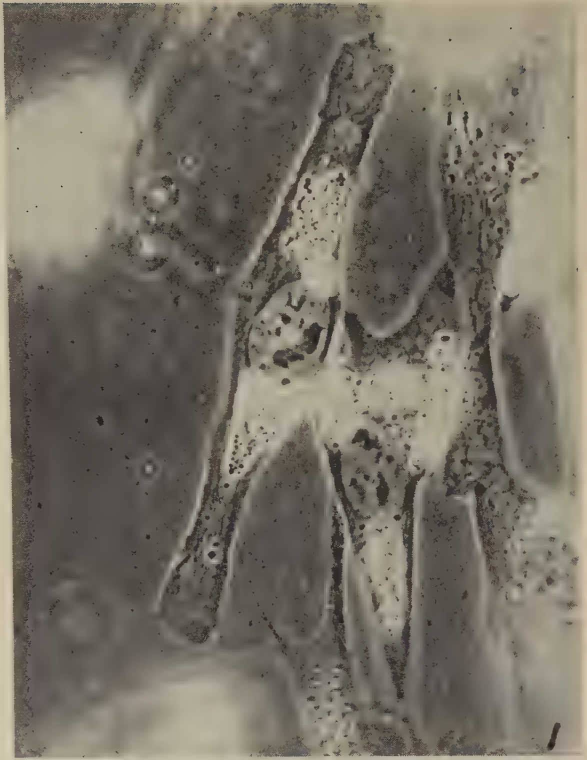
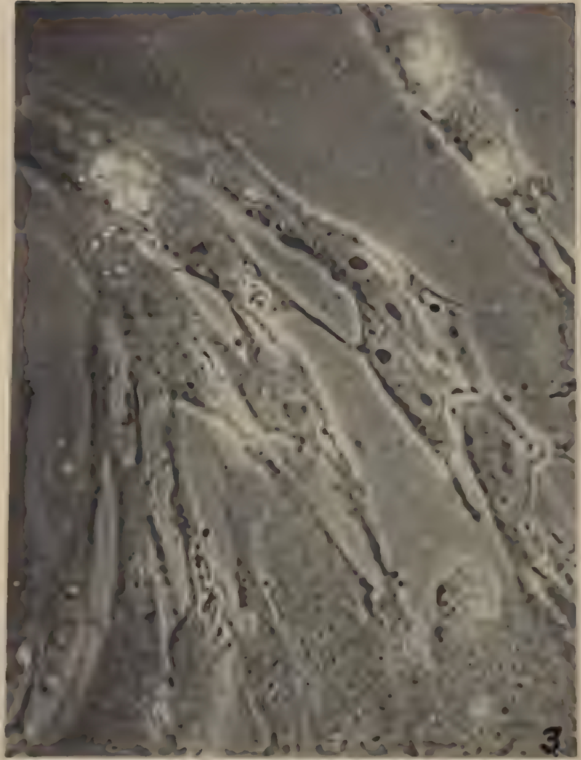


FIGURE 1. Cells of mouse sarcoma-180 in control culture. Phase contrast, 727 ×.

FIGURE 2. Cells of mouse sarcoma-180 damaged by six hours exposure to 0.1 mM 2,6-diaminopurine lactate. Phase contrast, 727 ×.



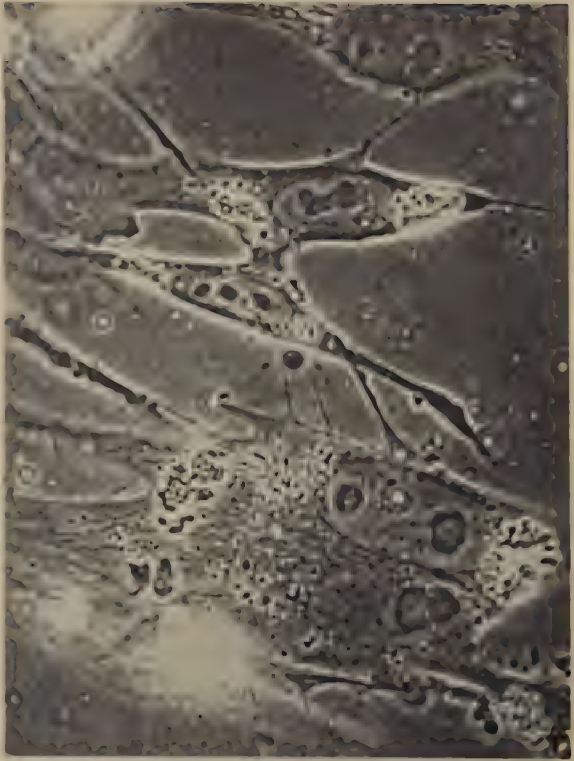


FIGURE 3. Fibroblasts of Ak mouse embryonic skin in control culture. Phase contrast, 727 ×.

FIGURE 4. Fibroblasts of Ak mouse embryonic skin relatively undamaged by six hours exposure to 0.1 mM 2,6-diaminopurine lactate. Phase contrast, 727 ×.

not show these drastic changes and appear comparatively unharmed. It is estimated that there was an approximately twentyfold difference in sensitivity of embryonic fibroblasts and sarcoma-180 cells to 2,6-diaminopurine (Biesele *et al.*, 1951).

However, treatment of sarcoma-180 in mice with purines that are differentially toxic in tissue culture, such as 2,6-diaminopurine, is often ineffective because of close approximation of the minimum effective level to a dose toxic to the animal (Stock, 1950). In the case of 2,6-diaminopurine, mammalian intestinal epithelium (Philips and Thiersch, 1949) and bone marrow (Cartwright *et al.*, 1950) are especially sensitive. In contrast, an agent such as 6-mercaptopurine,* which has given promising results with some complete cures in the treatment of sarcoma-180 in mice (Clarke *et al.*, 1953), shows little differential in its low toxicity in tissue culture.

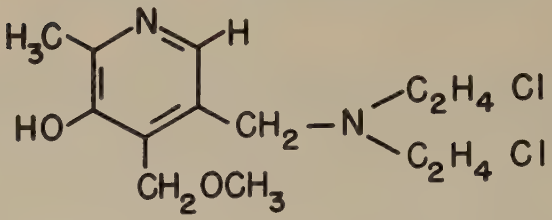
An interesting difference is found when one compares these two compounds with respect to their power to evoke differential mitotic inhibition. In our cultures, mitotic inhibition was nearly complete in sarcoma-180 cultures dosed with 0.1 millimolar 2,6-diaminopurine and in embryo skin cultures treated with 0.16 mM 2,6-diaminopurine. At half those concentrations, a fair amount of mitosis occurred in the cultures of the respective tissues (Biesele, Berger, Clarke, and Weiss, 1952). With 6-mercaptopurine, a greater differential in mitotic inhibition was obtained. TABLE 1 presents incidences of mitotic and pyknotic nuclei in sarcoma-180 and embryo mouse-skin cultures after one day of treatment with various concentrations of 6-mercaptopurine. Each entry is the average of counts on two cultures, except for the controls and 1.0 mM 6-mercaptopurine, which are based on four cultures each. A partial mitotic inhibition of sarcoma-180 cells extends over the concentration range from 4.0 to 0.5 mM, but the inhibition is lost at 0.1 mM. The skin cells show little depression in mitotic activity over this range.

Differential mitotic inhibitory power and the grosser cytological damaging propensities used in our routine screening have now been compared in forecasting the therapeutic activity of compounds. Eight of the 12 most effective compounds against sarcoma-180 in mice (Stock *et al.*, 1953), together with three

TABLE 1

MITOTIC AND PYKNOTIC NUCLEI IN CULTURES OF SARCOMA-180 AND MOUSE EMBRYO SKIN TREATED FOR ONE DAY WITH 6-MERCAPTOPURINE

6-Mercaptopurine	Mitotic		Pyknotic	
	(Nuclei per thousand)			
	S180	Skin	S180	Skin
0 mM	87	83	8	19
4.0	5	63	341	15
2.0	7	55	205	27
1.0	10	58	128	19
0.5	18	86	85	6
0.1	80	93	30	6



3-bis-(β-CHLOROETHYL)AMINOMETHYL-4-METHOXYMETHYL-5-HYDROXY-6-METHYL-PYRIDINE

FIGURE 5

TABLE 2
MITOTIC AND PYKNOTIC NUCLEI IN TISSUE CULTURES EXPOSED TO "PYRIDOXINE-NITROGEN MUSTARD" FOR ONE DAY

	Mitotic		Pyknotic	
	(Nuclei per thousand)			
	S180	Skin	S180	Skin
Control	84	96	9	12
3-bis-(β-chloroethyl) aminomethyl-4-methoxymethyl-5-hydroxy-6-methyl-pyridine				
0.2 mM	1	7	285	171
0.04	2	60	57	84

related compounds causing slight inhibition and three structurally related ineffective agents, have been tested in tissue culture.

The first agent, the so-called "pyridoxine-nitrogen mustard", or 3-bis-(β-chloroethyl)-aminomethyl-4-methoxymethyl-5-hydroxy-6-methyl pyridine* (FIGURE 5), was shown to be an effective agent by Sugiura and Stock (1950), by Stock, Buckley, Sugiura, and Rhoads (1951), and by Stock and Buckley (1951). In tissue culture, however, we obtained no differential damage with it. Damage was moderate to both sarcoma-180 and embryonic skin cells at 1.0 mM and negligible at 0.1 and 0.01 mM. A differential mitotic inhibition of sarcoma-180 cells was caused by it at 0.04 mM, as seen in TABLE 2. It may be pointed out in passing that cultures treated with a 5.0 mM concentration of the agent gave a perfectly normal mitotic picture. The agent had fixed the cells, and they were dead, as examination with phase contrast revealed. Having the stained cultures alone would have been misleading.

The nitrogen mustard HN2 was placed by Stock *et al.* (1953) in the ± category of slightly inhibitory compounds *in vivo*. HN3 had been reported by

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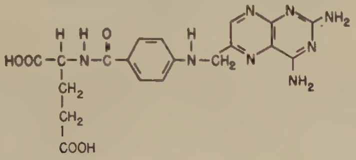
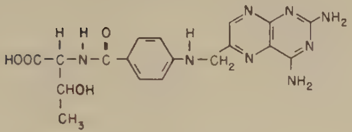
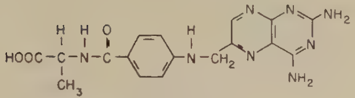
* Kindly supplied by Merck and Company Rahway, N. J.



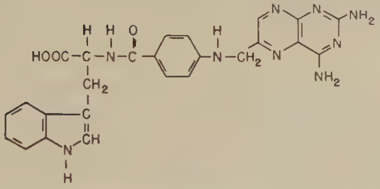
4-AMINOPTEROYLGLUTAMIC ACID



4-AMINOPTEROYLTHREONINE



4-AMINOPTEROYLALANINE



4-AMINOPTEROYLTRYPTOPHAN

FIGURE 6

Cornman and Ormsbee (1947) to cause a drop in mitotic activity in sarcoma-180 cultures at concentrations somewhat lower than those effective against fibroblast mitosis. The sarcoma cells were also more readily killed than fibroblasts.

In a set of five analogs of folic acid, 4-aminopteroylglutamic acid* was known to be inhibitory to sarcoma-180 *in vivo* (Stock, 1950; Stock and Buckley, 1951), as were 4-amino-N^{10}-methylpteroylglutamic acid† (Stock, 1950) and 4-aminopteroylthreonine* (Stock and Buckley, 1951). The related compound 4-aminopteroylalanine† was only slightly inhibitory *in vivo* (*Solid Tumor Screening Section*, unpublished), and 4-aminopteroyltryptophan‡ was ineffective (Stock *et al.*, 1953). Structures of these compounds with the exception of amethopterin are given in FIGURE 6.

Gradings of cellular damage in tissue culture with these five folic acid analogs are presented in TABLE 3. Damage is graded at none (0) through slight (+) and moderate (++) to extensive (+++). Much of each agent was in sus-

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* Kindly supplied by Lederle Laboratories.

† Kindly supplied by Calco Chemical Division, American Cyanamid Company.

TABLE 3

DAMAGE TO SARCOMA-180 AND EMBRYONIC MOUSE SKIN CELLS IN TISSUE CULTURE WITH FOLIC ACID ANALOGS

	Damage		
	S180	Skin	Diff'l.
4-aminopteroylglutamic acid			
10.0 mM	+++	+	++
1.0	+	+	0
0.1	0	0	0
4-amino-N^{10}-methylpteroylglutamic acid			
1.0 mM	+	+	0
0.1	0	0	0
4-aminopteroylthreonine			
10.0 mM	+++	++	+
1.0	+	+	0
0.1	+	0	+
4-aminopteroylalanine			
10.0 mM	+++	+++	0
1.0	0	0	0
0.1	0	0	0
4-aminopteroyltryptophan			
10.0 mM	+++	+++	0
1.0	++	+	+
0.1	0	0	0

pension at the 10.0 mM dose. A good differential damage was observed with 4-aminopteroylglutamic acid at the highest dose level, as had been previously observed with other mouse tumors (Stock, Biesele, *et al.*, 1950).

The phase-contrast photomicrographs of FIGURES 7 and 8 show the differential damage suffered by sarcoma-180 cells exposed to 5.0 mM 4-aminopteroylglutamic acid. Slight differential in damage was also observed with 4-aminopteroylthreonine. The slight differential with 4-aminopteroyltryptophan at 1.0 mM and the absence of differential with 4-amino-N^{10}-methylpteroylglutamic acid and 4-aminopteroylalanine are not in accord with results *in vivo*.

The results of mitotic and pyknotic counts in stained cultures that had been treated with aminopterin and its analogs are given in TABLE 4. Differentials in mitotic inhibition are evident throughout, as are differentials in the proportions of pyknotic nuclei. The sarcoma cells were more sensitive in every case. Their greater sensitivity to 4-aminopteroyltryptophan does not agree with ineffectiveness of this agent *in vivo*. The accumulation of mitotic metaphases, which occurred in many of the treated cultures, will be reported in detail elsewhere.

Triethylene melamine and two related substances (FIGURE 9) were also tested. The first agent, or 2,4,6-tris-ethylenimino-s-triazine,[5] was shown to inhibit sarcoma-180 in the mouse by Buckley *et al.* (1950). The second compound, 2-amino-4,6-bis-ethylenimino-s-triazine,† is also inhibitory *in vivo* (Buckley *et al.*, 1950) but is not included among the twelve most active agents given by Stock *et al.* (1953). The third of these agents, 2,4,6-tris-(2-vinyl-

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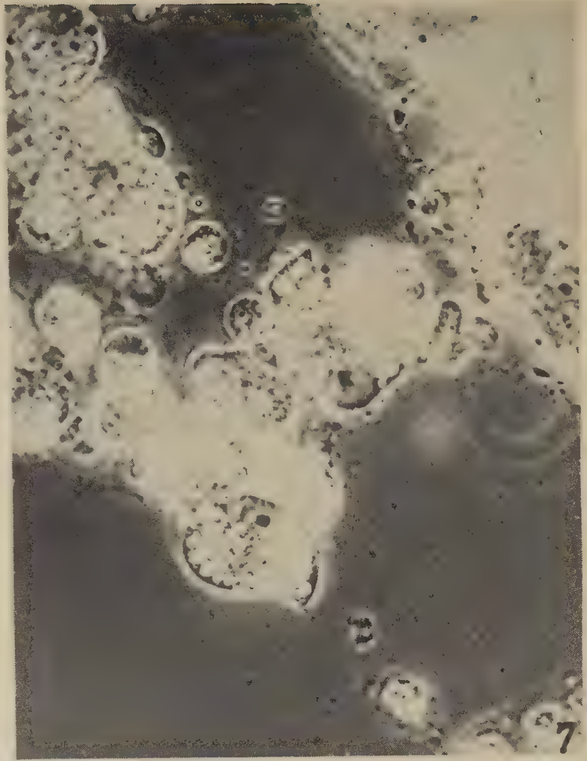
† Kindly supplied by the Calco Chemical Division, American Cyanamid Company.



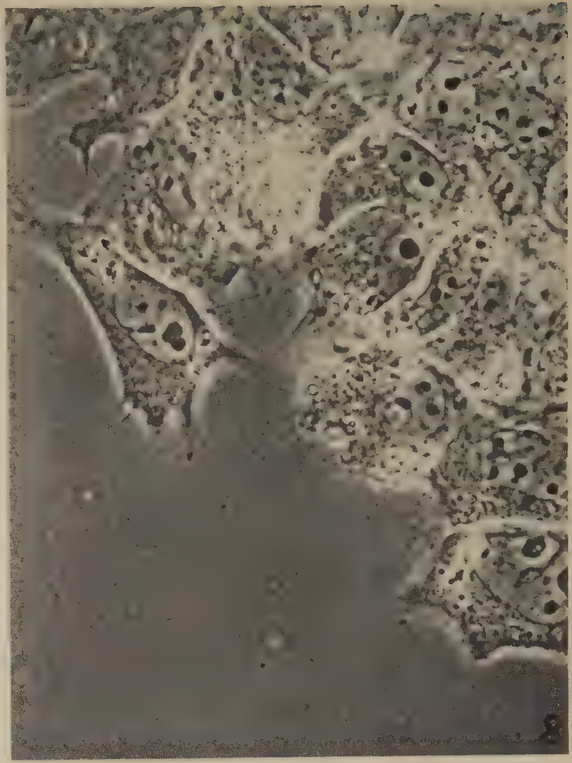


FIGURE 7. Sarcoma 180 cells injured by one-day exposure to 5.0 mM 4-aminopteroylglutamic acid. Note rounding and vacuolization. Phase contrast, 727 ×.

FIGURE 8. Embryonic mouse skin cells relatively unharmed by one-day exposure to 5.0 mM 4-aminopteroylglutamic acid. Phase contrast, 727 ×.

TABLE 4

MITOTIC AND PYKNOTIC NUCLEI IN SARCOMA-180 AND EMBRYONIC SKIN CULTURES TREATED ONE DAY WITH FOLIC ACID ANALOGS. EACH ENTRY IS THE MEAN OF READINGS ON THREE CULTURES

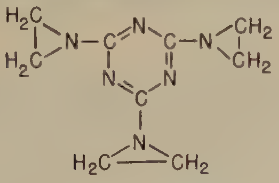
	Mitotic		Pyknotic	
	(Nuclei per thousand)			
	S180	Skin	S180	Skin
Control	84	96	9	12
4-aminopteroylglutamic acid				
5.0 mM	22	112	292	58
1.0	21	91	131	13
0.2	17	74	116	3
4-amino-N^{10}-methylpteroylglutamic acid				
1.0 mM	9	77	173	21
0.2	9	81	79	19
4-aminopteroylthreonine				
5.0 mM	3	88	520	149
1.0	36	74	159	8
0.2	15	47	49	14
4-aminopteroylalanine				
5.0 mM	4	46	351	132
1.0	12	114	148	13
0.2	17	102	90	15
4-aminopteroyltryptophan				
5.0 mM	0	1	999	273
1.0	4	40	273	23
0.2	20	69	73	32

aziridino)-s-triazine,† is ineffective in the mouse (*Solid Tumor Screening Section*, unpublished).

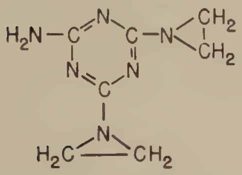
TABLE 5 gives assessments of damage in culture with these agents. A slightly greater damage to sarcoma-180 cells was obtained with the lowest concentrations of 2,4,6-tris-ethylenimino-s-triazine and 2-amino-4,6-bis-ethylenimino-s-triazine, but no differential was noted with the somewhat less toxic 2,4,6-tris-(2-vinylaziridino)-s-triazine. On closer examination with phase-contrast microscopy, the embryonic skin fibroblasts appeared more damaged than the sarcoma cells at an intermediate concentration of agent. FIGURES 10 and 11 illustrate the rounding and nuclear pyknosis that occurred in some of the fibroblasts in contrast to the healthy sarcoma cells.

The implication of these photographs was to some extent backed up by mitotic and pyknotic counts in stained cultures. TABLE 6 suggests a slightly greater sensitivity of the skin cells to 2,4,6-tris-(2-vinylaziridino)-s-triazine, but hardly any difference between sarcoma and skin cells in sensitivity to the other ethylenimino-s-triazines. It is to be noted that the vinylaziridino compound was considerably less active than the others.

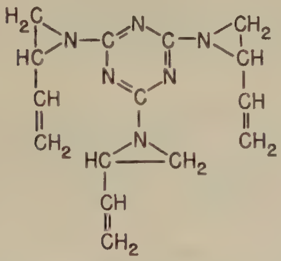
The last group of agents (FIGURE 12) also seems to depend for its activity on ethylenimine groups. In these, phosphate takes the place held by the s-triazine nucleus in the preceding set. Buckley *et al.* (1951) described the strong



2,4,6-tris-ETHYLENIMINO-s-TRIAZINE



2-AMINO-4,6-bis-ETHYLENIMINO-s-TRIAZINE



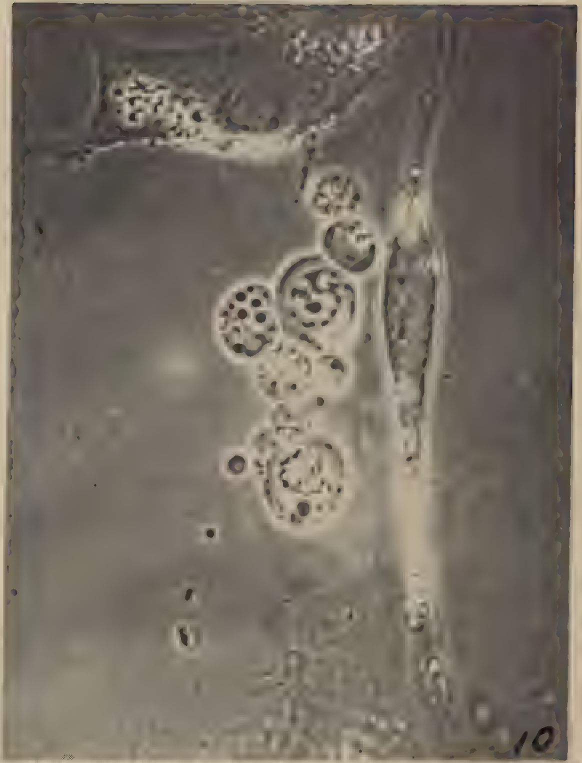
2,4,6-tris-(2-VINYLAZIRIDINO)-s-TRIAZINE

FIGURE 9

TABLE 5

GRADINGS OF DAMAGE TO SARCOMA-180 AND EMBRYONIC SKIN CELLS FROM ETHYLENIMINO-S-TRIAZINES IN TISSUE CULTURE

	Damage		
	S180	Skin	Diff'l.
2,4,6-tris-ethylenimino-s-triazine			
1.0 mM	+++	+++	0
0.1	+++	+++	0
0.01	++	+	+
2-amino-4,6-bis-ethylenimino-s-triazine			
1.0 mM	+++	+++	0
0.1	+++	+++	0
0.01	+++	++	+
2,4,6-tris-(2-vinylaziridino)-s-triazine			
1.0 mM	+++	+++	0
0.1	+	+	0
0.01	0	0	0



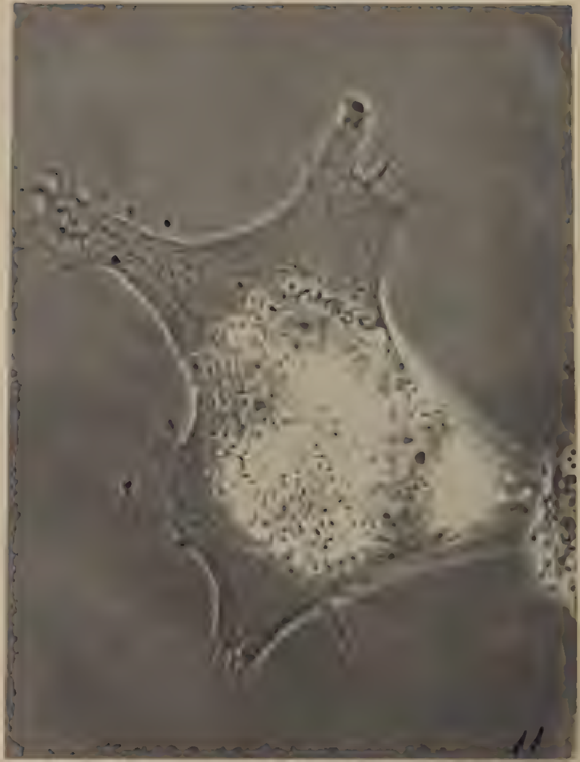


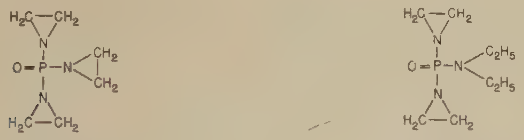
FIGURE 10. Fibroblasts of embryonic mouse skin, some rounded and pyknotic, after one-day exposure to 0.1 mM 2,4,6-tri-[illegible]-s-triazine. Phase contrast 727 X.

FIGURE 11. Healthy [illegible] cell after one-day exposure to 0.1 mM 2,4,6-[illegible]-s-triazine. Phase contrast, 727 X.

TABLE 6

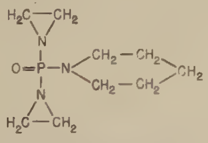
MITOTIC AND PYKNOTIC NUCLEI IN CULTURES OF SARCOMA-180 AND EMBRYONIC MOUSE-SKIN CELLS TREATED WITH ETHYLENIMINO-S-TRIAZINES

	Mitotic		Pyknotic	
	(Nuclei per thousand)			
	S180	Skin	S180	Skin
Control	95	105	27	25
2,4,6-tris-ethylenimino-s-triazine				
0.05 mM	0	0	496	535
0.01	2	9	188	78
0.002	22	28	39	61
2-amino-4,6-bis-ethylenimino-s-triazine				
0.01 mM	0	0	388	303
0.002	9	9	44	44
0.0004	32	41	51	33
2,4,6-tris-(2-vinylaziridino)-s-triazine				
0.5 mM	3	0	173	323
0.1	53	40	13	45
0.02	96	64	25	31

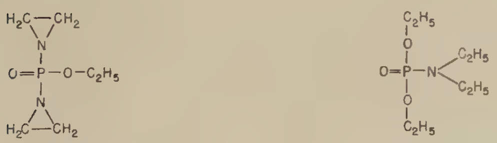


N,N',N"-TRIETHYLENE PHOSPHORAMIDE

PHOSPHORIC ACID, DIETHYLAMIDE DIETHYLENIMIDE



N-PENTAMETHYLENE-N', N"-DIETHYLENEPHOSPHORAMIDE



ETHYL N,N'-DIETHYLENE DIAMIDOPHOSPHATE

DIETHYL N,N-DIETHYL-AMIDOPHOSPHATE

FIGURE 12

TABLE 7

MITOTIC AND PYKNOTIC NUCLEI IN CULTURES OF SARCOMA-180 AND EMBRYONIC MOUSE-SKIN CELLS TREATED WITH TRIETHYLENE PHOSPHORAMIDE AND RELATED AGENTS

	Mitotic		Pyknotic	
	(Nuclei per thousand)			
	S180	Skin	S180	Skin
Control	91	122	12	2
N,N′,N″-triethylene phosphoramide				
1.0 mM	0	0	567	605
0.2	1	6	196	166
0.04	13	8	49	39
Phosphoric acid, diethylamide diethylenimide				
1.0 mM	2	1	310	468
0.2	4	12	129	113
0.04	10	36	75	36
N-pentamethylene-N′,N″-diethylenephosphoramide				
1.0 mM	1	1	342	455
0.2	4	11	185	55
0.04	17	16	32	24
Ethyl N,N′-diethylene-diamidophosphate				
5.0 mM	0	0	835	729
1.0	1	6	234	170
0.2	18	11	53	19
Diethyl N,N-diethylamido-phosphate				
5.0 mM	50	60	24	3
1.0	85	75	47	5
0.2	101	95	9	9

inhibitory action of N,N′,N″-triethylene phosphoramide† and phosphoric acid, diethylamide diethylenimide† on sarcoma-180 in mice. The list of twelve compounds most inhibitory to sarcoma-180 *in vivo* also includes N-pentamethylene-N′,N″-diethylene phosphoramide† (Stock *et al.*, 1953). Slight inhibitory activity is shown by ethyl N,N′-diethylene-diamidophosphate† (*Solid Tumor Screening Section*, unpublished). Diethyl N,N-diethylamidophosphate is inactive* (Stock *et al.*, 1953).

In tissue culture, no differential damage to sarcoma-180 cells was noted with any of these compounds. The four compounds bearing ethylenimine groups caused extensive damage at 10.0 mM, and generally moderate damage at 1.0 mM, except for ethyl N,N′-diethylene-diamidophosphate, which caused no damage at that concentration. Diethyl N,N-diethylamidophosphate appeared harmless.

Similar results were obtained with counts of mitotic and pyknotic nuclei. TABLE 7 presents evidence for a mitotic inhibition of equal extent in both

* Kindly supplied by Monsanto Chemical Company.

† Kindly supplied by the Calco Chemical Division, American Cyanamid Company.

tissues treated with the ethylenimine-agents. Again, diethyl N,N-diethylamidophosphate had the least adverse effect on the cultures.

The only correlation with *in vivo* results to be noted with these compounds and, to some extent, with the ethylenimino-s-triazines, is that compounds less toxic in tissue culture were less active against sarcoma-180 in the mouse, while the phosphoramides more toxic in culture were more inhibitory to the tumor in the animals.

Discussion

It is evident from these results that our assay procedures for agents differentially toxic to neoplastic cells are often not to be relied upon to give accurate indications of results with a given agent in the animal, even when the same neoplasm is employed. When one considers what may happen to a therapeutic agent in an animal, with possibilities of excretion, detoxification, metabolic degradation, or localization to particular sites in the body, it is surprising that there should be occasional agreement of *in vivo* results with those *in vitro*. Nevertheless, tissue-culture assay may provide important clues in cancer chemotherapy. With refinement of methods and understanding of what to look for with given types of agents, perhaps more significant assays in particular families of chemicals can be carried out in tissue culture.

Changes in assay technique might go, for instance, in either of two directions. The culture system might be made even more simple. Elimination of the plasma clot would provide more direct access of the test chemicals to the cells (Pomerat, 1951). Provision of a defined synthetic medium would allow for better control of metabolite-antimetabolite interactions, of importance with agents of certain types. Better control would also result from the use of stabilized continuous cultures, which would avoid the considerable cytolysis occurring in explants fresh from the animal. Maintenance of normal cells in vessels separate from those in which the neoplastic cells are cultured would remove possible protective effects of the normal cells (Jacquez, Barclay, and Stock, 1952). All these changes would make the test system even less like the animal, and agreement between results *in vivo* and *in vitro* might be even less likely.

The other direction would make the test system in culture more like the animal. One might grow explants of various organs, *e.g.*, liver, whose cells might be expected to modify some test chemicals, together with the neoplastic cells (Biesele and Jacquez, 1954). The obvious extension of this method is to make the assay directly in animals. If this procedure is impossible because of a limited supply of chemical, a tissue-culture test could still be used.

The findings of any tissue-culture assay are to be interpreted with caution. The results are not necessarily applicable beyond the particular combination of tumor, normal tissue, and culture conditions employed. The results would be so applicable only if there should be some unique difference or differences between all neoplastic cells and all normal cells, and if this hypothetical difference were susceptible to chemical attack. If no such unique difference exists, we must continue to base our hopes for successful chemotherapy on the manipulation of incidental metabolic differences which exist between certain types of neoplastic cells and certain types of normal cells as well as between different strains of neoplastic cells themselves. Elucidation of such differences, as by means of experimentation with antimetabolites, would appear to be a more reasonable use of tissue culture in the field of cancer chemotherapy than the routine screening of chemicals on a purely empirical basis.

Summary

A tissue-culture procedure for the assay of chemicals for antineoplastic activity is described. It is essentially a parallel comparison of toxicity of agents to neoplastic and embryonic cells of the mouse. Results of this assay may or may not agree with the known effectiveness or lack of effect of a series of agents on sarcoma-180 in the mouse. In some cases, determination of differential mitotic inhibition in tissue culture made for better agreement with results *in vivo*.

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DOCTOR ROBERT D. BARNARD (*Research Consultant, Rystan Company, Inc., Mount Vernon, New York*): The discordance shown to exist by Doctor Biesele between tissue culture and animal assay of "antineoplastic" substances might be amplified in light of his own explanation of the correlation absence; that vagaries of distribution, metabolism, and excretion in the whole animal might preclude effective concentrations at the site of the *in vivo* malignancy. The described tissue-culture technique was an expedient for simplification of the system, permitting "screening" delineation of promising cytotoxic agents. The whole technique entailed concomitant testing against normal tissue so that an approximation of therapeutic index might be obtained.

Considering the ultimate goal of this screening, the therapeutic index might best be calculated from the ratios of cytotoxic action against neoplastic, as compared with hemopoietic marrow and/or intestinal mucosa cell cultures, instead of an arbitrarily chosen "normal" tissue because, as we shall see, susceptibility of the former nonmalignant tissues has been the prepotent limiting factor of cytotoxic and radiomimetic drugs so far elucidated.

Doctor Biesele's explanation of the tissue culture versus *in vivo* efficacy discordance might further be evaluated by a critical examination of the premises comprising the induction of this sequence: (1) the problem of cancer is fundamentally a cellular problem; (2) antineoplastic substances which selectively poison the neoplastic cell should be sought by direct tissue-culture technique; so that (3) promising agents or their modifications may then be tested in tumor-bearing animals; and (4) this yield, in turn, should finally be "screened" on humans afflicted with cancer.

The facile epicheirema may harbor some logical fallacy; its constituent premises must therefore be scrutinized in numerical order.

(1) The cancer problem is fundamentally a cellular one, but the inability of

a dyspeptic to elaborate digestive enzymes and that of a uremic to eliminate nitrogenous wastes are also cellular problems.

Premise (2) could, therefore, beg the question, and there are other reasons for refusing to derive it either from (1) or from our general fund of information. What (2) begs from (1) is that the *direct* cytotoxic approach is the only reasonable or feasible one to the problem of established human cancer. Clinical experience serves as contradiction. Oncolytic or oncostatic drug modalities devoid of sensible cytotoxic or radiomimetic effects are known. Oral administration of mycin antibiotics, or the crude fermentation residues from which these antibiotics are obtained, administered in doses below those yielding a sensible systemic level—let alone a cytotoxic one—have reversed the courses of some human cancer patients. Androgen or estrogen therapy, once thought to be operative solely on derivatives of heterosexual tumors and to be examples of direct cellular restraint, may not actually be so. Not only may metastases from breast or ovarian carcinomae detumesce during testosterone administration, but some from lung, intestinal tract, or prostate do likewise. Similar results have been observed during metalloporphyrin administration, though the materials injected are completely devoid of any cytotoxic action.

(4) Unfortunately the limitation on animal-tumor screening has been so generally recognized that one tends to omit or ignore it and to take the results from (2) directly to (4), thus depriving the human of possible safeguards posed by the *in vivo* screening. For the fact remains that indiscriminate application of the antifolics and nitrogen mustards did materially shorten lives rendered more uncomfortable by their exhibition. This is particularly poignant because adjudications of the leukemia-lymphoma group of neoplasia as diseases of adaptation have validated the clinical impression that even the acute forms of these leucoblastoses are potentially and actually remissible. The hematomyelaplasia produced by cytotoxic and radiomimetic drugs in subjects with these diseases has been, in my experience, less reversible than the condition they purport to control. Contentions have been raised and cannot be summarily dismissed that remission attributed to folic-acid antagonists or nitrogen mustards were either coincidental or actually due to the routine, conjoint administration of mycin antibiotics [Canad. M.A.M. **13**, 614 (Dec.) 1950]. True, these contentions remain debatable, but so, for that matter, does the validity of the restrictive approach to the cancer problem through the narrowly defined species of chemotherapy emerging from the Sloan-Kettering prospectus. I am pleased that the latter has been formalized for us, because that formalization has opened the door for discussion around its ultimate channeling to "screenings" on human beings. And because, here, moral as well as scientific implications are involved, such discussion should be especially deliberate.

METABOLIC AND CHEMOTHERAPEUTIC INVESTIGATION OF HUMAN GLIOBLASTOMA *IN VITRO**

By Margaret R. Murray, Edith R. Peterson, Erich Hirschberg, and J. Laurence Pool

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The work here described was undertaken with the intent of inquiring into the metabolic pattern, or the biochemical equipment, of the human glioblastoma multiforme with the ultimate aim of gaining a measure of chemotherapeutic control over this rapidly and widely infiltrating neoplasm. No spontaneous transmissible animal glioblastoma was available for a pilot study, but it was felt, in any case, that since very wide differences in ability to cope with carcinolytic agents exist between species as closely related as the rat and the mouse,† there was great advantage in proceeding at once to work with human tissues. Biopsies from operation, maintained in tissue culture, afforded the material for this investigation of the susceptibility of the human glioblastoma multiforme to potential lytic agents.

No data were available on the susceptibility of gliomas to any chemotherapeutic agent. In our preliminary experiments it was thought expedient to choose as potential inhibitor a compound which was selectively toxic to epithelial tumors rather than to those of fibroblastic affinity, since we were dealing in the glioblastoma with cells that were descendents of neurectoderm. And since the guanine analog, 8-azaguanine (FIGURE 1A), had been reported to inhibit a variety of carcinomas in the mouse, rabbit, and rat,‡ but to be generally ineffective against sarcomas; and since it had been the subject of many metabolic studies in animals (mentioned by Gellhorn *et al.* 1954), this compound was chosen.

At the same time, by homogenate studies, Hirschberg, Kream, and Gellhorn (1952) were adducing evidence that in both normal and neoplastic tissues an important factor governing susceptibility or resistance to 8-azaguanine lay in the capacity of the tissue in question to transform this guanine analog by deamination to 8-azaxanthine (FIGURE 1B; PLATE 5, NUMBERS 19, 20), a completely harmless derivative. Tissues that are resistant are high in deaminase activity, and those that are susceptible carry out little deamination. Tested by this homogenate method, human glioblastoma tissue was found to be virtually devoid of capacity to deaminate azaguanine, whilst normal adult brain exhibits the highest azaguanine deaminase activity of any tissue so far studied. By chromatographic analysis of feeding-fluids from tissue cultures, as shown by Hirschberg *et al.* (1953), human glioblastoma tissue *in vitro* gives results that are entirely consistent with the homogenate studies, and perceptibly more sensitive. Its deaminating capacity is vanishingly small.

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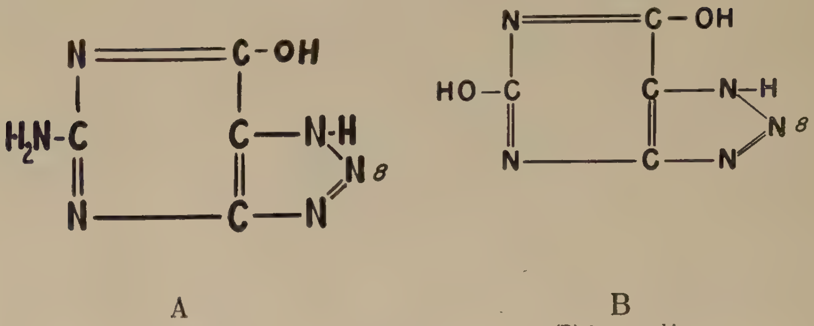
* This work was supported by the John Gunther, Jr. Fund and by Grant C-1752 from the National Cancer Institute of the National Institutes of Health.

† *Cf.* Biesele *et al.* 1951.

‡ Gellhorn *et al.* 1950; Sugiura *et al.* 1950; Kidder *et al.* 1951.



(A) 8-azaguanine (B) 8-azaxanthine

FIGURE 1.

With this indication of metabolic peculiarity on the part of the human glioblastoma, and with the demonstration that functional tests by the tissue culture and homogenate methods corroborate each other on this lack of enzymatic action, it was thought feasible to utilize the method of tissue culture to investigate further the mechanism by which azaguanine acts upon this tumor. Because of its purine structure, it was natural to inquire whether it is able to replace some normal constituent of nucleic acids, and to test this putative action by the simultaneous administration of such compounds. To this end, the two purines adenine and guanine, and their nucleosides, were selected: adenine because of its very general utilization by animal cells; and guanine because of the inconsistencies that seem to exist with regard to its exogenous utilization among species and among tissues both normal and neoplastic (Hamilton, 1953), as well as because of its structural similarity to azaguanine. These results are reported in *Observations*, *Part I*, of this communication.

Though it has been demonstrated both by results reported here and in preliminary studies by the authors (Hirschberg *et al.* 1953) that the human glioblastoma is inhibited *in vitro* by 8-azaguanine, a group of extrinsic factors combine to counteract this compound's effectiveness when it is administered clinically by the usual intravenous route. Among these factors, summarized by Gellhorn (1953), are the relative insolubility of this purine analog and the high deaminating capacity of normal liver tissue, which join to prevent the maintenance of an effective blood level for the drug. In addition, certain normal tissues such as skin possess only low deaminase activity and may show toxic effects from slight or transient exposure.

Consequently, in view of the problems inherent in the clinical application of azaguanine, it became desirable to screen related compounds as well as other agents of interest against this tumor in the hope of uncovering a more effective and satisfactory carcinocide. To this end, benzimidazole and six congeners* of itself or of azaguanine as well as the aliphatic compound Myleran have been tested on tumors from six different patients. These preliminary observations in a long-range screening program are incorporated in *Observations*, *Part II*.

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* Synthesized by Doctor Carl T. Bahner, Carson-Newman College, Jefferson City, Tenn.

Material and Method. Portions of human tumors (diagnosed as glioblastome multiforme by members of the Neuropathology Staff) were obtained at operation through the cooperation of the staff of Neurological Surgery. It was the custom to make cultures from these specimens on the same day that they were obtained. Occasionally, however, the material was kept in a moist chamber (prepared by placing a few drops of balanced salt solution in a tightly closed bottle along with the specimen) at approximately 4° C. overnight. This latter treatment was in no way deleterious.

Stock solutions 0.1 M, of 8-azaguanine, 8-azaxanthine, and the normal purines,* were prepared by heating a suspension of the solid in one third the final volume of 0.5 N NaOH until a clear solution was obtained. This solution was then partially neutralized (to pH 8) with 1 N HCl and made up to volume. A control solution containing NaOH alone at the concentration present in the experimental groups was included in each series.

The solutions of congeners of azaguanine and of benzimidazole, tested as potential chemotherapeutic agents in the second half of this account, were prepared in similar manner. For Myleran, however, it was necessary to use propylene glycol as a solvent, constituting one third the desired volume of an 0.1 M stock solution. A control containing propylene glycol alone in feeding solution was used in this case. These agents were tested on tumor cultures at final concentrations of 0.02 M and 0.005 M in feeding solution. In each drug series one group receiving 8-azaguanine at 0.005 M was included for comparison of kind and degree of effect.

The tumor tissue was grown for six to eight weeks in roller tubes according to the method of Gey and Gey (1936) before being transferred to Maximow slides for the experiments. Here the double-coverslip, lying-drop method was used, with a basic feeding medium composed of human placental serum, beef-serum ultrafiltrate, chick-embryo extract, and chicken plasma, in which the experimental agents used in *Part I* were incorporated so as to be present in final concentrations of 0.005 M (with the exception of guanine, which would not remain in solution at this level and was therefore employed at 0.0025 M and 0.0033 M). Feeding solutions containing the agents to be tested were renewed four times (following washing of the culture in Simms' balanced saline) over a 10-day observation period. During this period they were observed daily (always by the same person [ERP]) at a magnification of 300 times and rated for degree of damage, according to a standard set of criteria, which follows:

Criteria for Toxicity Rating

(1) Inhibition of migration	moderate—severe
(2) Inhibition of mitosis	
(3) Nuclear changes (pyknosis, fragmentation)	moderate—severe
(4) Cytoplasmic changes (blebbing, vacuolation, granulation)	moderate—severe

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* The authors are indebted to Doctor J. M. Ruegsegger of the Lederle Laboratories for the supply of 8-azaguanine, and to Doctor Jacob Kream for the 8-azaxanthine. The normal purines and their ribosides were obtained from Schwarz Laboratories.

(5) Blunting of cell processes and rounding of cells — moderate—severe

Severe damage in any 3 of the above listings, to some degree reversible, yields a +++ rating.

Irreversible damage, leading to death of culture, receives a ++++ rating.

Explants from tumors of eleven different patients were used in these series of four experiments involving adenosine, adenine, guanosine, and guanine. From 100 to 200 cultures composed each of the four series. From two to five different tumors were used in each series; three tumors were used in more than one series.

Each of the four series contained four groupings, of which the following is typical:

Series 1. Adenosine: (a) adenosine alone; (b) adenosine + azaguanine (equimolar); (c) azaguanine alone; and (d) control.

For permanent record, some of the cultures were photographed living, by the use of phase-contrast microscopy. All were fixed at the conclusion of the experiments, in either absolute methyl alcohol followed by Jenner-Giemsa staining, or in Zenker's fluid followed by phosphotungstic acid haematoxylin.

Tissue controls. The use of human fetal brain cultures as tissue controls in the preliminary azaguanine tests is discussed by Hirschberg, Murray, Peterson *et al.* (1953).

Observations. Part I

Progressively severe damage, lethal within 10 days for the majority of the tumors tested, was produced consistently by 8-azaguanine.

The group of tumors classified as glioblastoma multiforme (Bailey, 1932) evolve a variety of cell types *in vitro.* An early and accurate description of the behavior of this tumor (to which the authors can add little) was published in 1933 by Russell and Bland; this description was accompanied by a time-lapse motion picture (made with the aid of R. G. Canti) which is still extant, depicting the form and behavior of a variety of human gliomas in tissue culture. It has been remarked that cultivation of cells of nervous derivation in an environment where they are released from pressure and allowed to migrate freely as in a tissue culture, tends to intensify their distinguishing morphological characteristics (Murray and Stout, 1954). An example of this tendency appears in NUMBER 1, PLATE 1, where the rayed form of these living cells surpasses any distinguishing mark to be found in sectioned material.

In the authors' experience, one or two cell types usually predominate in a given tumor; consequently there is considerable variation from tumor to tumor in growth rate and also in reaction to compounds tested. The more rapidly growing cell types may be classified as spongioblasts, oligodendroblasts, and undifferentiated spindle cells. Astroblasts may be divided functionally into a rapidly growing type which bears some resemblance to a polar spongioblast (NUMBER 10, PLATE 3), and a less active type which develops either a fibrous rayed or a feathery form such as the astroblasts of Fañana (NUMBERS 7, 9).

All the cell types cultivated may be multinucleate. Some give the impression of nuclear fragmentation, though this process has not been observed directly. Mitoses are present, though not always frequent. Where the tumor is characterized by a large proportion of multinucleate cells, as in NUMBER 2, mitoses are rare or absent. A considerable proportion of the areolae surrounding the explants from these tumors is produced by migration rather than multiplication of cells; but a rapidly dividing type is usually also a rapidly migrating type.

With this great variation in cell form one might expect also a varied response to test agents. This variation takes the form of greater susceptibility to azaguanine among the spongioblasts, oligodendroblasts, and spindle cells, and a considerable resistance in the slow-growing astroblastic type. Among different tumors and different areas of one tumor there are substantial variations in susceptibility to inhibiting compounds. In a general way, susceptibility is correlated positively with growth rate.

Series 1. Adenosine (*two tumors tested*).—Adenosine administered alone was entirely nontoxic to one of the two tumors tested, and very slightly toxic to the other (RAN). In both cases it afforded excellent protection against azaguanine when the two compounds were administered simultaneously in equal molarity (0.005 M). NUMBERS 1 to 6, PLATES 1 and 2, are from the same tumor, designated KAR from the patient's name. NUMBERS 1 and 2 are untreated controls. NUMBER 3 shows early stages of azaguanine damage (withdrawal of processes, rounding and granulation of cells) still reversible; NUMBER 4 represents irreversible and lethal damage. NUMBER 5 shows an undamaged culture, to which adenosine has been administered, and NUMBER 6 a culture shielded by adenosine from azaguanine damage. Protection is complete, and mitoses are observed.

Series 2. Adenine (*three tumors tested*).—Adenine by itself at 0.005 M is fairly toxic. It does not cause withdrawal of processes, as shown in NUMBER 7, PLATE 2, but it inhibits both growth and migration. Of the three tumors tested, one showed extensive damage different in kind but equal in severity to azaguanine damage in the same tumor (KO) as shown in TABLE 1. However, in this tumor simultaneous exposure to both the normal purine and the analog was less damaging than exposure to either alone. NUMBERS 7 to 9, PLATES 2 and 3, show effects in LIP, a tumor which was not greatly damaged by adenine alone and was well, though not completely, protected against azaguanine.

Series 3. Guanosine (*three tumors tested*).—The toxicity of this riboside varies substantially from tumor to tumor. For the tumors to which it is not toxic it affords protection as great as that of adenosine; the tumor RAN, which was used in both experiments, was well protected by both compounds. Guanosine, however, can show an inhibiting effect equivalent to the milder forms of adenine damage, but not necessarily for the same tumor. Tumor KO, which was severly damaged by adenine, is essentially uninjured by guanosine, and is afforded nearly complete protection by it (NUMBERS 10 to 12, PLATE 3).

Series 4. Guanine (*four tumors tested*).—The administration of guanine was complicated by its low solubility in the vicinity of neutrality. Unless introduced into the feeding fluids at greater alkalinity than that required by the

Table 1

Human Glioblastoma In Vitro: Reversal of Inhibition

(Schematic Summary)

Series	Reversing agent alone		8-azaguanine + reversing agent damage	8-azaguanine alone damage	Control (NaOH) damage
	Tumor	Damage			
Adenine M/200	KO	+++	++±	+++	0
	CA	+±	+	+++±	0
	LIP	+±	+	+++±	0
Adenosine M/200	RAN	±	±	+++±	0
	KAR	±	±	+++±	0
Guanine M/400	HE	±	+	+++	±
	TON	±	+	+++	±
	CAT	±	+	+++	±
	HU	±	+	+++	±
Guanosine M/200	KO	+	+	+++	0
	CA	+	+	+++	0
	RAN	±	±	+++	0

It will be noted that tumors KO, CA, and RAN are tested for more than one agent. Tumors KO and CA merit different susceptibility ratings for adenine, but respond similarly to guanosine. RAN receives the same rating for guanosine as for adenosine.

other three compounds, it rapidly crystallized out. It could not be kept in solution at a pH which would permit growth of the controls, in concentrations greater than 0.0025 M. Number 14, plate 4, shows a living culture which has ingested guanine crystals appearing in the medium after a concentration of 0.005 M had been introduced.

Guanine is only slightly toxic when in solution, and not at all toxic when present in crystalline (presumably inert) form. When administered as one half mol per one mol of azaguanine (0.0025 M/0.005 M) it affords good protection—greater than adenine—and only slightly less than adenosine at equimolarity (numbers 13 to 15, plate 4). A 0.0025 M concentration of azaguanine, however, is not lethal. The above data on reversal of toxicity are summarized in table 1.

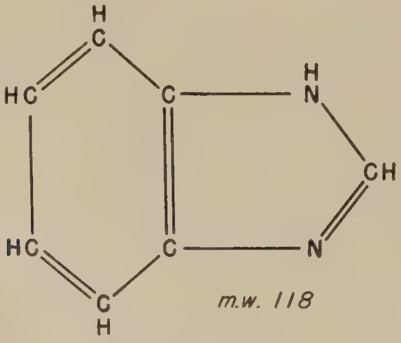

Figure 2. Benzimidazole

Observations. Part II

In the screening program designed to uncover other agents potentially more active than azaguanine or more favorable as regards solubility and side effects, eight compounds (FIGURES 2 to 8) have so far been evaluated, on explants derived from a total of six different tumors. These have been given preliminary testings at 0.02 M and 0.005 M in feeding solution on groups of eight explants, with four groupings (a total of 32 explants) to a series—an adequate number for eliminating ineffective compounds at these concentrations. Each

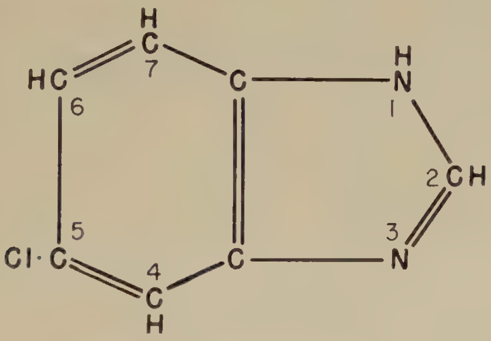

FIGURE 3. 5 (or 6)-Chlorbenzimidazole

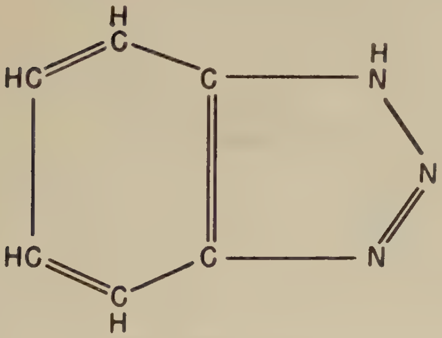

FIGURE 4. Benzotriazole

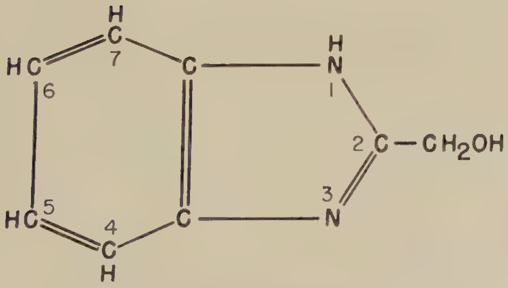

FIGURE 5. 2-Hydroxymethylbenzimidazole

series includes: (1) compound X at 0.02 M; (2) compound X at 0.005 M; (3) azaguanine at 0.005 M; and (4) control containing the solvent alone. Agents shown to be effective will be examined more thoroughly on additional tumors, with wider concentration range and greater numbers of explants.

Of the eight agents tested in this preliminary manner four have been found ineffective, and four effective in varying degree when applied directly to human glioblastomas *in vitro*. In TABLE 2, effectiveness is rated in comparison with azaguanine.

Benzimidazole is a mild inhibitor of glioblastoma, about one quarter as effective as azaguanine. Administered at 0.02 M it is lethal (NUMBERS 16 to 18, PLATES 4 and 5), but at 0.005 M produces only very mild inhibition. It does not act to bring about retraction of processes, which is one of the early signs of azaguanine damage.

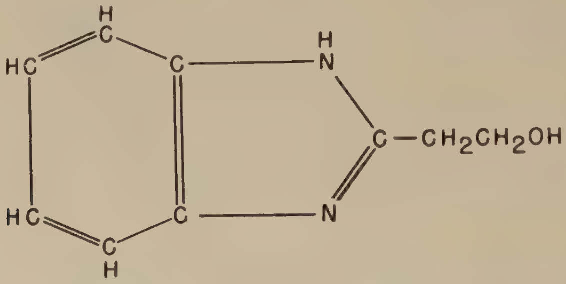

FIGURE 6. 2-(1-Hydroxyethyl)benzimidazole

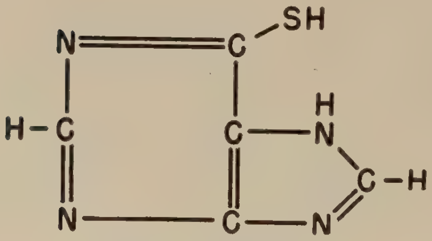

FIGURE 7. 6-Mercaptopurine

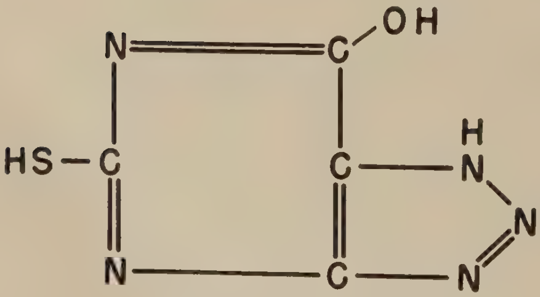

FIGURE 8. 5-Thiol-7-hydroxy-1H-v-triazolopyrimidine

TABLE 2

INHIBITION OF HUMAN GLIOBLASTOMA IN VITRO

Ineffective	Effective	Degree
	8-Azaguanine	+++
Benzotriazole	Benzimidazole	+
2-Hydroxymethylbenzimidazole	5 (or 6)-Chlorbenzimidazole	++++
2-(1-Hydroxyethyl)benzimidazole	6-Mercaptopurine	+
5-Thiol-7-hydroxy-triazolopyrimidine	1,4-Dimethanesulfonyloxybutane (Myleran)	+++

5 (or 6)-Chlorbenzimidazole, on the contrary, is more effective than azaguanine at equal molarity. This agent, tested on three different tumors, does not bring about retraction of processes even in the most branching of astrocytic forms (numbers 21 to 24, plate 6). The highly distinctive response of the tumor material to this compound appears to be a form of nuclear extrusion, as illustrated in number 23 (*cf.* comment by Doctor Lessler). Number 24 shows the effects of azaguanine on the same tumor.

6-Mercaptopurine is of effectiveness approximately equal to benzimidazole; *i.e.*, lethal at 0.02 M and only slightly inhibitory at 0.005 M (numbers 25, 26, plate 7). This compound, as reported by Hamilton *et al.* (1954), appears to pass the blood-brain barrier, but is very rapidly metabolized at multiple sites.

1,4-Dimethanesulfonyloxybutane. This aliphatic chain-compound $CH_3SO_2OCH_2CH_2CH_2CH_2OSO_2CH_3$, purveyed commercially as Myleran, was introduced from the Chester Beatty Institute as an inhibiting agent for myeloid leukemia (Galton, 1953; Haddow and Timmis, 1953). Since it was found by Gellhorn, Peterson, *et al.* (1954) to be inhibitory to mouse glioma 26, it was tested also on these human tumors. This compound appears to be at least as effective as azaguanine (numbers 27, 28, plate 7) but the reading is complicated somewhat by toxicity of the solvent, propylene glycol. In the group containing Myleran at 0.01 M, toxicity begins to be registered within 48 hours; in the control group containing propylene glycol only, inhibition becomes apparent at six days. Until the drug can be tested using another solvent, one cannot exclude synergism as a possibility.

Discussion

These observations on glioblastoma multiforme make it clear that for this human tumor, developing spontaneously from fundamentally the same tissue at the same general site but in different individuals, susceptibility to a given chemical agent varies on all these counts. This generalization applies not only to analogs or other potential carcinolytic agents, but to "normal" constituents of nucleic acids administered exogenously. Response to the agent varies from tissue type to tissue type within the tumor, and from area to area. It also varies among tumors from different individuals. Over and above these variants, a basic factor in variability appears to be the growth or division-rate of the tissue. There is little reason to believe that malignant human tumors of tissue origin other than glioblastoma are essentially less variable in these respects; the gamut of cell forms that can be run by liposarcoma or rhabdomyosarcoma for example, is as great if not greater than that which we see in this tumor. The point is borne out by the records of individual variation in response to chemotherapeutic agents which have accumulated in practically every clinic administering chemotherapy.

It is suggested that because these variabilities are an essential part of the problem of treating human neoplasms, it is desirable to study as fully as possible under experimental conditions the human tumors, which are the ultimate object of any chemotherapeutic program. Admitting the necessity and great value of animal screening projects, and the inadequacy of any one approach to the complexities of tumor metabolism, it is believed nevertheless that the tissue culture of human tumors affords an important means of bridging the gap between animal tests and clinical trial. This approach has a potential utility which is as yet little explored: once a spectrum of potential agents were assembled, a biopsy could be tested in about ten days, and the most effective inhibitor selected for that individual patient's tumor. That such procedure could not cover every contingency is well understood, and it is exemplified by the authors' report on discrepancies between *in vivo* and *in vitro* effects of 6-mercaptopurine on mouse glioma 26 (Gellhorn, Peterson, *et al.* 1954)—to cite only one such observation. Nevertheless, it is believed that the advantage of this method for evaluating individual susceptibility is very considerable.

Summary

The inhibitory effects of 8-azaguanine 0.005 M on human glioblastoma multiforme in tissue culture are shown to be reversed in large degree by adenine, adenosine, and guanosine at equal molarity, and by guanine at one half this molarity. Each of these normal nucleic acid constituents is toxic in some degree to this tissue. When administered alone, adenine is the most toxic, adenosine the least.

Benzimidazole and six other compounds, congeners of itself and of 8-azaguanine, were tested on this human tumor for carcinolytic action. Of these, benzimidazole and 6-mercaptopurine are slightly inhibiting as compared to azaguanine; 5- (or 6)-chlorbenzimidazole is more inhibitory than azaguanine. The aliphatic compound Myleran (1,4-dimethanesulfonyloxybutane) is equivalent to azaguanine in inhibiting effect on this tissue *in vitro*.

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Discussion of the Paper

Doctor Albert Hartzell (*Boyce Thompson Institute for Plant Research, Yonkers, N. Y.*): We have been interested in the possible application of insect tissue culture in the assay of insecticides in a similar manner to that in which mammalian tissue culture is used in the assay of carcinolytic agents and drugs. Using classical methods (chick embryo extract and chicken plasma medium), we have been able to cultivate insect brain and connective tissues from American roach nymphs, *Periplaneta americana* (L.), and wax moth larvae, *Galleria mellonella* (L.). Explants were obtained by vivisection under CO_2 anesthesia and washed repeatedly in Ringer's solution. These tissues have lived in hanging drop cultures until the 12th transplant, or about three months. The cultures were incubated at a temperature of 30 ± 1° C., and new media added weekly. No attempt to buffer the cultures was made except through the addition of media. Penicillin was added to inhibit growth of possible bacterial contaminants. The results obtained to date are preliminary. It has not been determined positively, for example, whether the "growth" in our cultures is attributable to proliferation and migration of cells, or to mitotic division, although Gavrilov and Cowes,* using a somewhat similar method with mosquito larva tissue in malarial research have reported mitotic division of esophagus tissue in culture.

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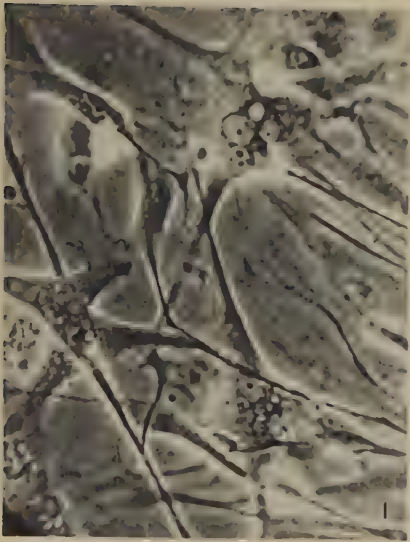
* 1941 Ann. parasitol. humaine et comparée. 18: 180–186.

Plate 1, Number 1. KAR control, living, phase contrast, 225 ×. Number 2. KAR control, fixed, stained by Jenner-Giemsa method, J-G, 225 ×. Number 3. KAR early damage (reversible) by azaguanine, J-G, 225 ×. This stain emphasizes basophil accumulations in the cytoplasm. Number 4. KAR lethal azaguanine damage, J-G, 225 ×.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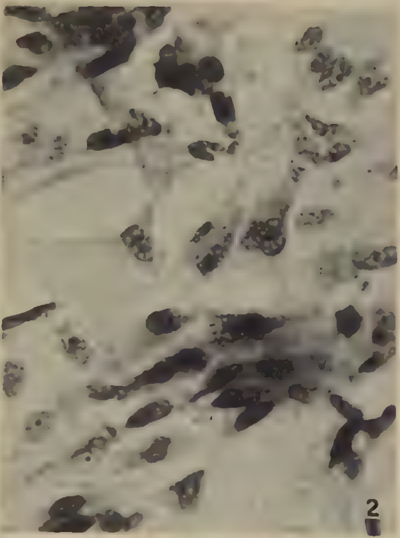
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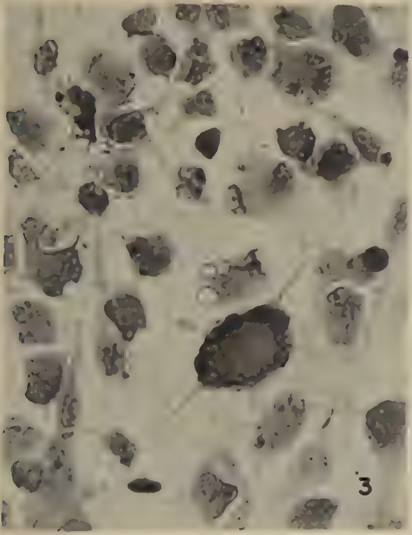
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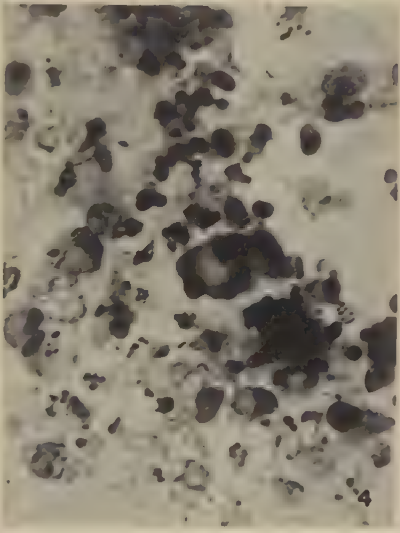
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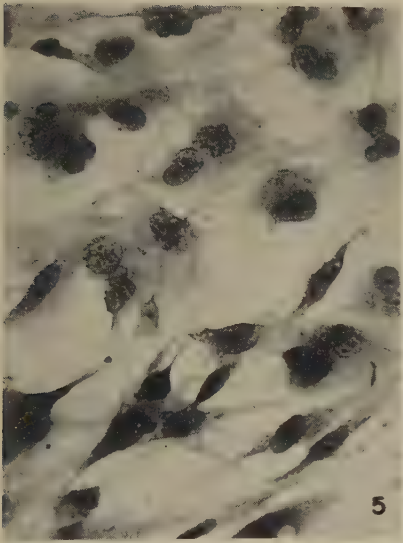
Plate 2, Number 5. KAR adenosine alone, J-G, 225 ×. Number 6. KAR azaguanine + adenosine, each at 0.005 M, J-G. 225 ×. Number 7. LIP adenine alone, J-G, 225 ×. Number 8. LIP azaguanine, J-G, 225 ×.



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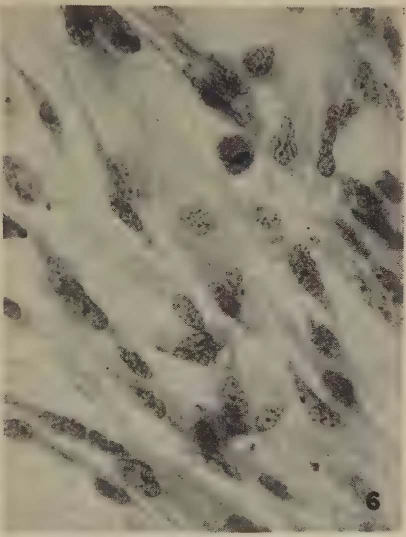

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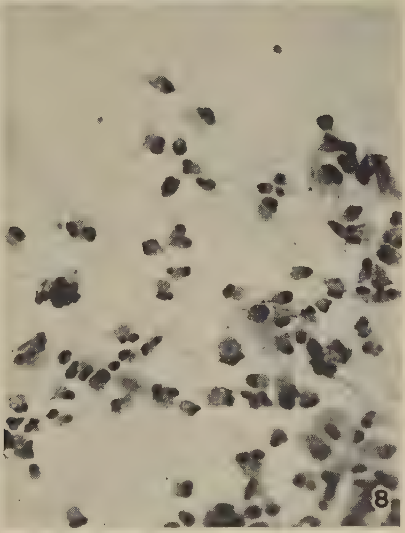

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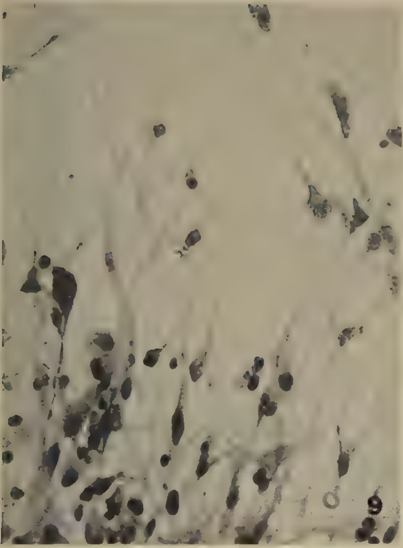
Plate 3, Number 9. LIP azaguanine + adenine, each at 0.005 M, J-G, 225 ×. Number 10. KO guanosine alone, J-G, 225 ×. Number 11. KO azaguanine, J-G, 225 ×. Number 12. KO azaguanine + guanosine, each at 0.005 M, J-G, 225 ×.



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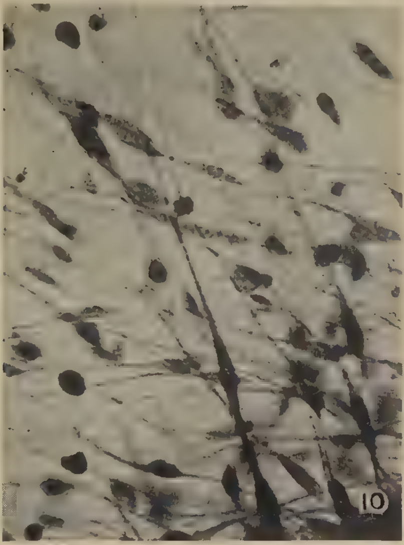

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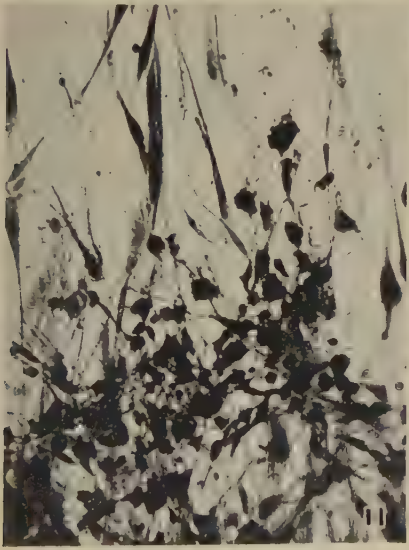

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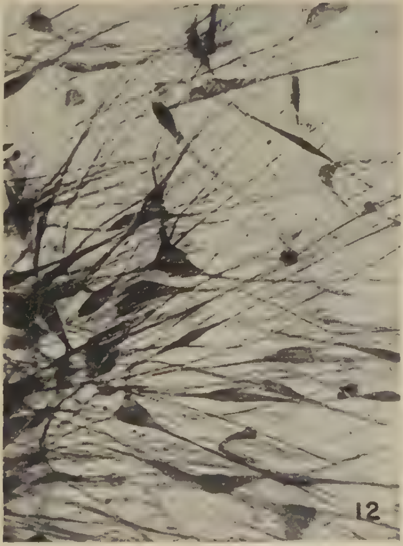

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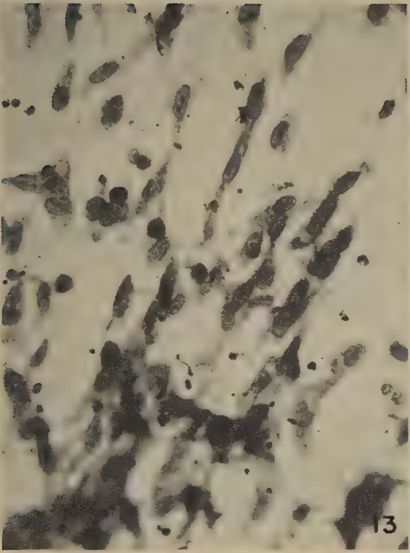
Plate 4, Number 13. TON azaguanine, 0.0025 M, J-G, 225 ×. Number 14. TON azaguanine, 0.0025 M, guanine, 0.005 M, J-G, 225 ×. Number 15. TON azaguanine + guanine, both 0.0025 M, J-G, 225 ×. Number 16. STR control, J-G, 225 ×.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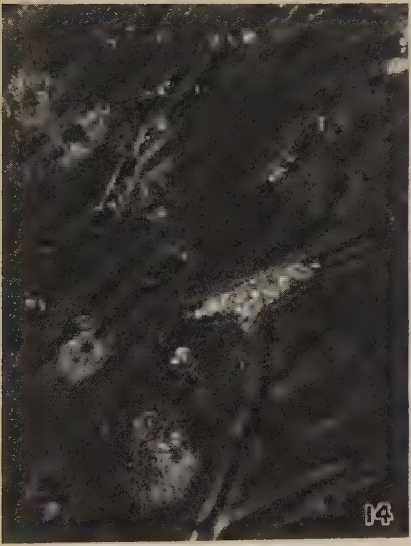
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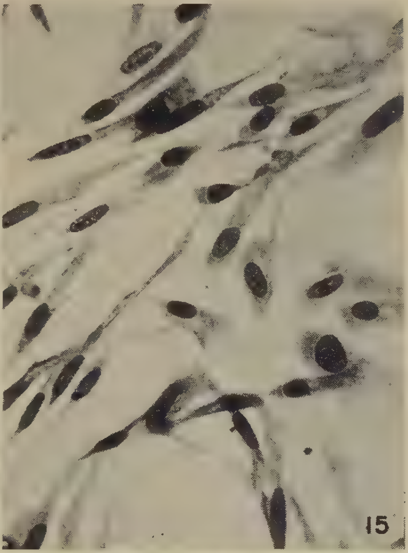
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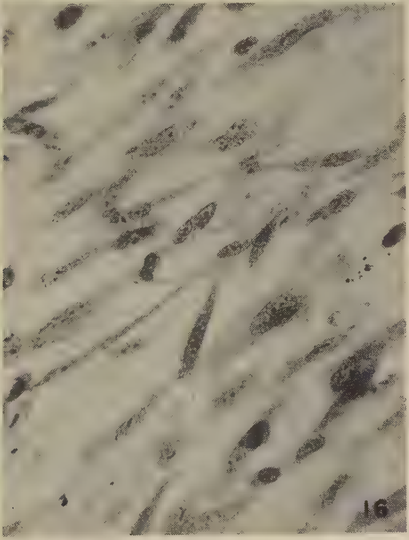
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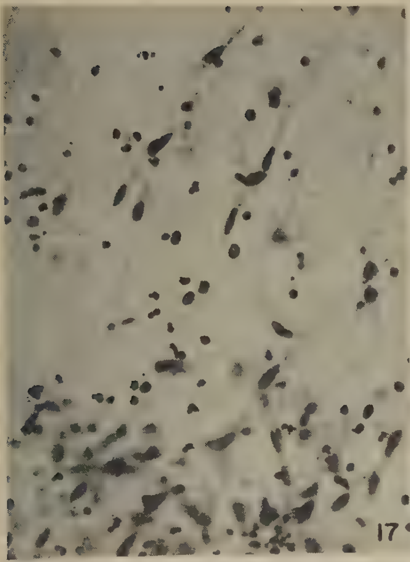
Plate 5, Number 17. STR benzimidazole, 0.02 M, J-G, 225 ×. Number 18. STR azaguanine, 0.005 M, J-G, 225 ×. Number 19. MC azaxanthine, 0.005 M, phosphotungstic acid haematoxylin (PTA), 225 ×. Number 20. MC azaguanine, 0.005 M, PTA, 225 ×.



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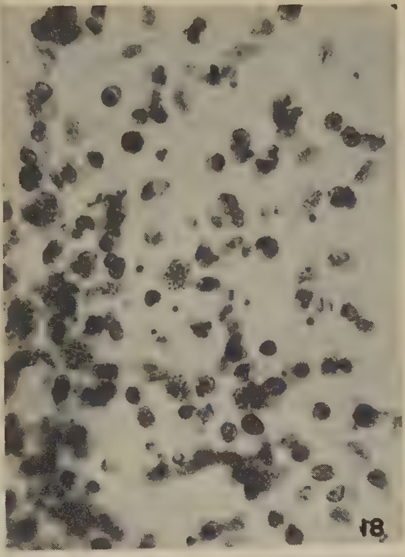

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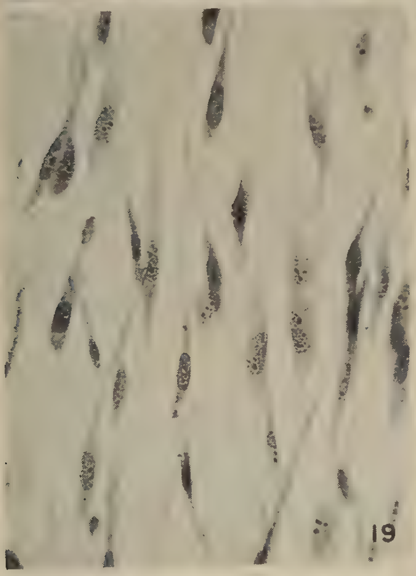

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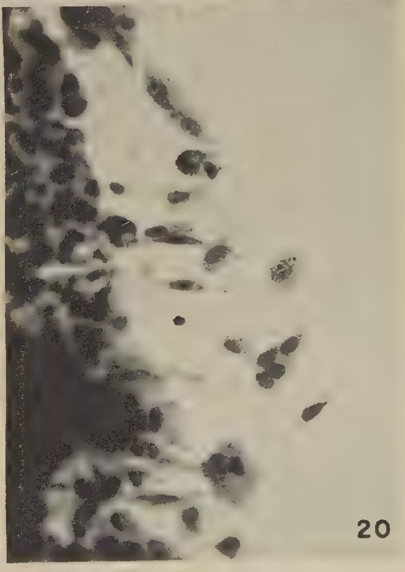

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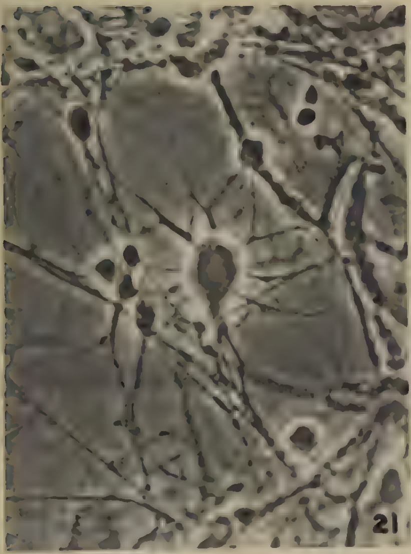
Plate 6, Number 21. GL control, living, phase contrast, 300 ×. Number 22. GL chlorbenzimidazole 0.005 M, living, phase contrast, 300 ×. Cytoplasmic masses from which nuclei have been extruded. Number 23. GL chlorbenzimidazole, 0.005 M, J-G, 225 ×. Nuclear extrusion. Number 24. GL azaguanine damage, 0.005 M, living, phase contrast, 300 ×.



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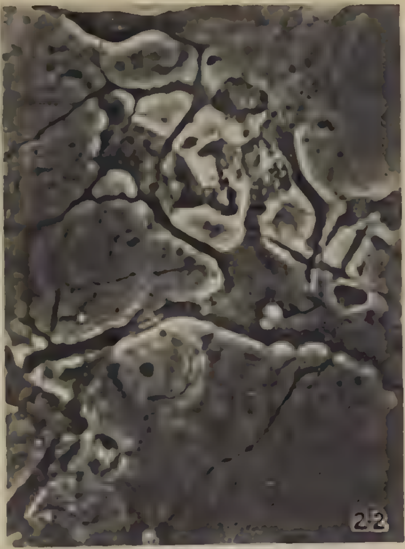

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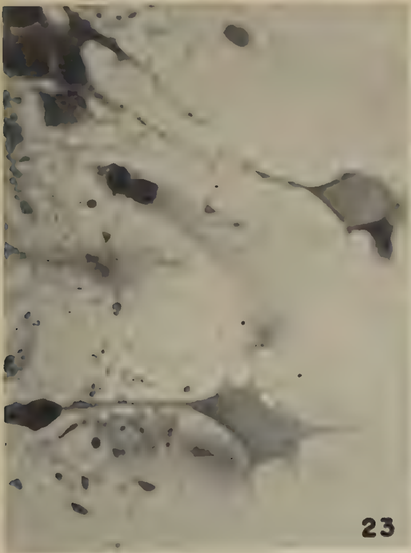

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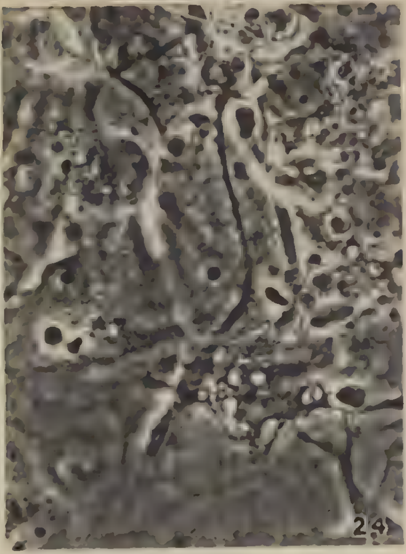

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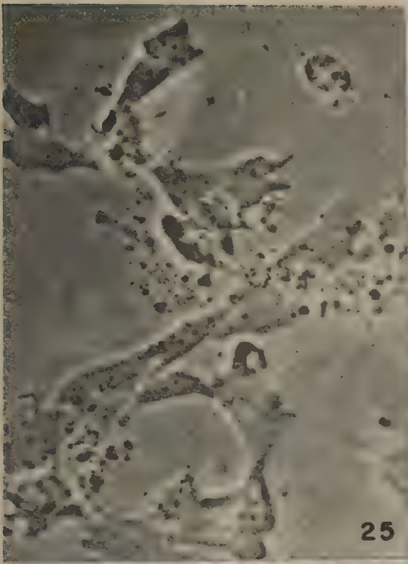
PLATE 7, NUMBER 25. RAN 6-mercaptopurine, 0.02 M, severe damage, phase contrast, 300 ×. NUMBER 26. RAN control, living, phase contrast, 300 ×. NUMBER 27. GB propylene glycol control, living, 5 days, phase contrast, 300 ×. NUMBER 28. GB Myleran, 0.01 M, 5 days, severe damage, phase contrast, 300 ×.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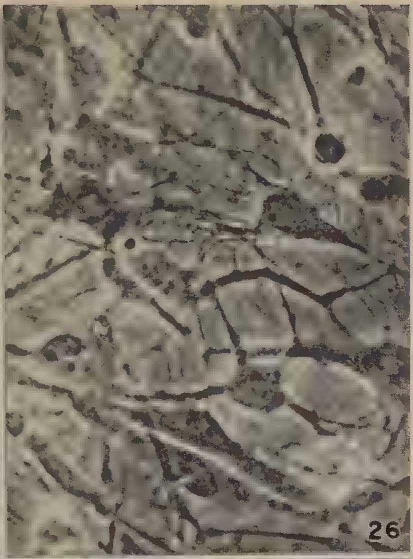
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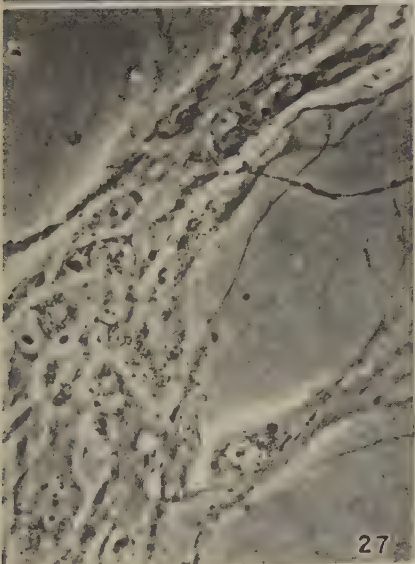
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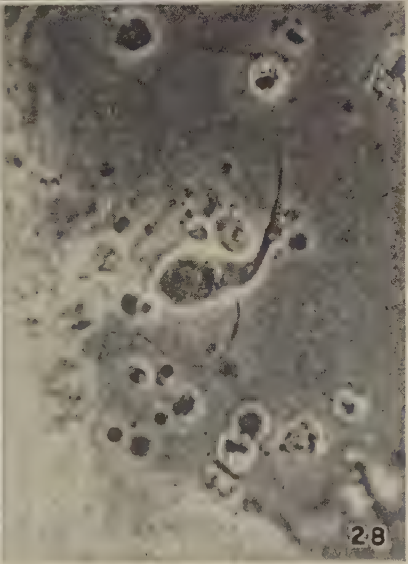
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A COMPARISON OF *IN VIVO* AND *IN VITRO* TESTS FOR ANTINEOPLASTIC ACTIVITY OF EIGHT COMPOUNDS

By P. A. Eichorn, K. V. Huffman, J. J. Oleson, S. L. Halliday, and J. H. Williams

Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

Introduction

The use of cultured cells as a means of assaying compounds for their antineoplastic activity has been employed in a number of studies (Willmer 1939, Cameron 1944, Cornman 1944, [a, b] 1947 and 1950–1951, Pomerat 1945, Norris 1948, Ormsbee 1949, and Gunz 1950). In accordance with the general application of the screening of compounds, other investigators, Stock 1951, Antikajian 1951, Schrek 1953 and, notably, Biesele in 1951 and in three publications in 1951[a, b, c], have tested a significant number of substances on colonies of normal and tumor animal cells cultured *in vitro*.

The tissue culture studies of Ormsbee (1949) with podophylin, Stock (1951) with folic acid, and Biesele (1952[c]) with 2-azaadenine, revealed a greater toxicity of these substances for tumor cells than for normal cells. Subsequent *in vivo* tests of these materials, however, did not substantiate these tissue-culture conclusions of selectivity or effectiveness. The identification by Cameron (1944) of heptanal sodium bisulfite as exerting a selective toxicity on human mammary tumor cells was substantiated fairly well by Strong (1938) on a spontaneous mammary tumor in mice. In general, it may be said that the relatively few comparisons of activity of substances against tumors in animals and tissue culture have shown but little correlation.

At a session of the Second National Cancer Conference held in March 1952, Doctors C. C. Stock of the Sloan Kettering Institute and Alfred Gellhorn of the Francis Delafield Hospital both emphasized the lack of correlation between the observed effects of compounds on animal tumors and on human tumors. As an alternative, they proposed either the use of human patients or possibly tissue culture as a means of screening antineoplastic compounds. The hesitancy with which they proposed the use of tissue culture is understandable in view of the previous poor correlation noted in using animal tumors and tissue culture, and because the tissue culture technique admittedly imposes an artificial environmental condition on the cells.

On the other hand cancer can be considered as, fundamentally, a cellular problem. This being the case, we felt that we possessed presumptive incentive for experimenting once again with this technique. The possible advantages that could accrue from the establishment of the validity of tissue culture as a screening technique have been frequently catalogued. Of these advantages however, a most appealing one is the possibility of screening antitumor substances against cultures of human tumor cells. Of fundamental importance in the use of this technique, however, is the validity of such a method.

It seemed to us that a reasonable approach to a substantiation or disproof of the validity of using cultures would be to compare the effect that certain

TABLE 1

LIST OF SUBSTANCES TESTED ON MOUSE TUMORS, MAMMARY 72J AND BRAIN 8110 IN VIVO AND IN VITRO

Compound	Molec. wgt.	Solubility
(1) 6-Dimethylamino-9-[3′-(L-phenylalanylamino)-β-D-ribofuranosyl] purine	441	Soluble
(2) N^5-benzyl-2-pyrrolidone-5-carboxamide	218	Soluble
(3) N,N′,N″ triethylenethiophosphoramide	145	Soluble
(4) O-methyl-L-tyrosine	195	Soluble
(5) N-N′-Di-p-chlorobenzyl malonamide	351	Insoluble
(6) 1-Carbamyl-4-*o*-chlorobenzoylpiperazine	267.7	Insoluble
(7) N-(3-oxapentamethylene)-N′,N″-diethylenethiophosphoramide	233.3	Insoluble
(8) 6-Mercaptopurine	170	Insoluble

compounds have on certain tumors in animals and on the same tumor types *in vitro*. Mindful of the discrepancies which had accumulated from past endeavors we hoped that in our repetition of this work we might observe some fundamental criterion or essential factor in the procedure which had been previously overlooked.

Materials and Method. In all, eight chemical substances were evaluated on one type of normal tissue and two types of animal tumors. These substances, as shown in TABLE 1 may be divided into two groups; the first four chemicals are either soluble, or easily put into solution; the remaining four substances are almost completely insoluble.

Selection of these compounds was based on their *in vivo* activity against two animal tumors. This activity was entirely unknown to the people conducting the tissue culture tests. It is important to note that the results reported in this paper were not based on the selection of eight compounds from a host of others tested in tissue culture, but rather on the first eight consecutive compounds issued for testing.

These eight compounds were tested at various concentrations on cultures of normal embryonic mouse skin, and on the mouse mammary 72J tumor and the mouse brain tumor 8110, both of which are carcinomas. These tumors were selected for testing for two reasons: first, the mammary 72J tumor, better known as the Bittner mammary tumor, is of spontaneous origin and frequently contains two types of cells. In these respects it differs from the brain tumor 8110, which was originally chemically induced and possesses cells morphologically alike. Second, a representative amount of data had been accumulated on the activity of compounds on these tumors during the years of extensive screening against tumors conducted at Lederle. It is worthy of note that *in vivo* tests have shown the 8110 tumor to be highly resistant to the activity of most chemical substances.

Basically, our method of assay followed conventional methods of tissue

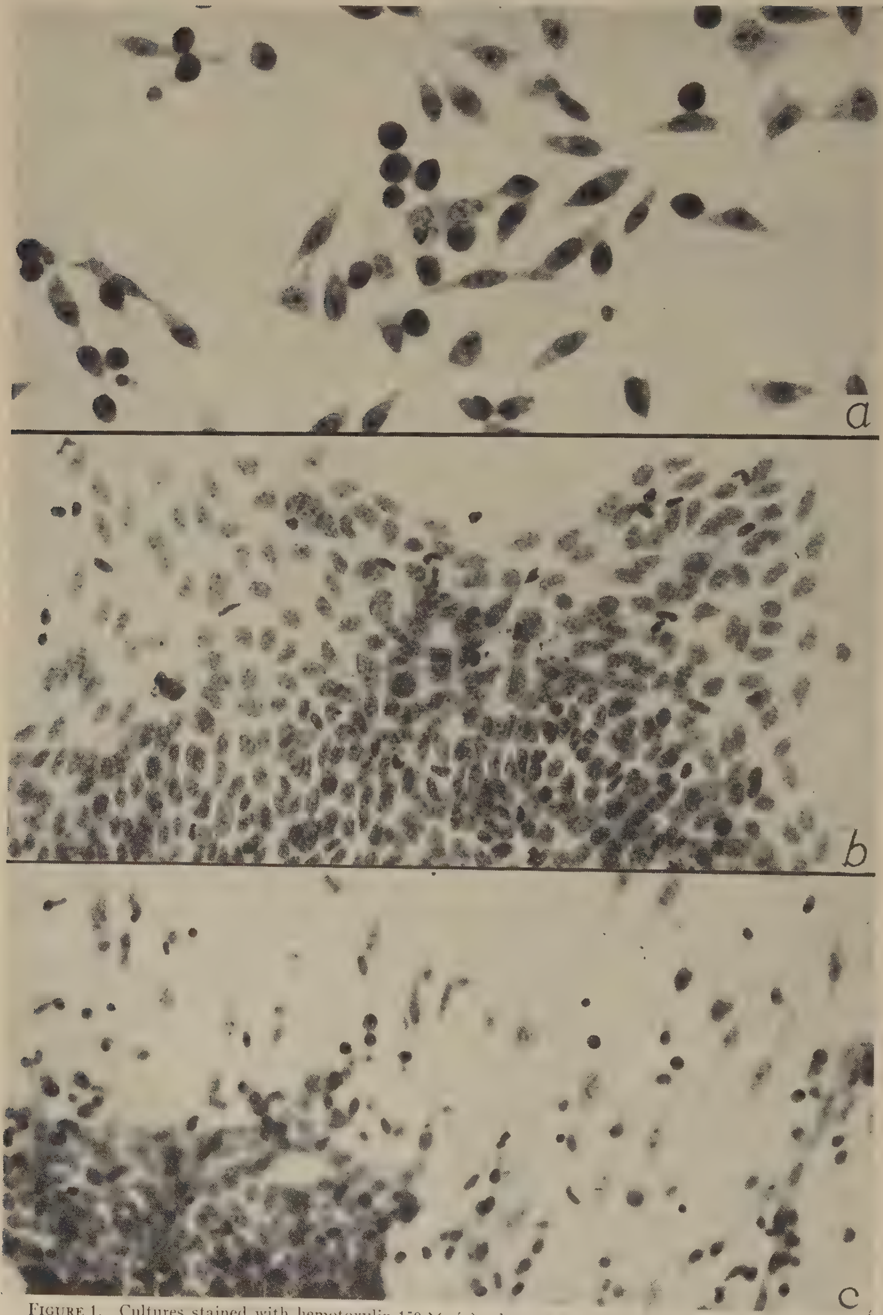

FIGURE 1. Cultures stained with hemotoxylin 150 ×: (*a*) colony of 8110 brain tumor cells cultured *in vitro*, (*b*) sheet of mammary J tumor cells cultured *in vitro*; (*c*) Sheet of mammary J tumor cells associated with fibroblast-like cells emanating from explant cultured *in vitro*.

culture. The effective concentrations of the chemical substances were first approximately obtained by a preliminary screening for toxic levels on embryonic chick cells cultured in roller tubes. Once these levels were established, various concentrations of the substances were added to cultures of embryonic mouse skin as a normal control, and to cultures of mouse mammary J tumor and to the 8110 brain tumor.

FIGURE 1, (*a*), (*b*), and (*c*) illustrate the types of cells seen in cultures of these two tumors. The 8110 tumor is composed of cells that are distinct, clear-cut, and rarely, if ever, accompanied by fibroblasts (FIGURE 1(*a*)). These cells extend into the clot from the explant much more rapidly than do the J cells. The mammary J cells extend out as epithelial sheets (FIGURE 1(*b*)). In many cultures of the tumor a few to many fibroblast-like cells are also present (FIGURE 1(*c*)).

In application our method has been essentially as follows: fresh tissues for each experiment were removed from the respective animals and small explants (approximately 1 $mm.^3$) were implanted in clots in roller tubes, usually four to five fragments per tube. Each tube contained only one type of tissue. In most experiments, at least two tubes of each tissue to be tested, containing eight to ten fragments, were used for each concentration. All cultures were initially nourished in a horse serum, chick embryo extract medium for 24 to 48 hours, depending upon the type of tissue. At the time of testing, all tubes were read for satisfactory extension of cell colonies; those showing poor extension or undesirable cell morphology were eliminated. The growth-promoting medium was then removed and replaced with a 0.004 per cent concentration of bovine blood albumin containing the various concentrations of substances to be tested. These cultures were then incubated for another 48 hours at which time they were fixed, stained, and read.

Our evaluation of the effects of these compounds on the cells follows in general the norms established by Pomerat (1951). The concentration affecting the least amount of damage to the cells is identified as a 1+ or the LID, that is, least injurious dose. Progressive amounts of damage are recorded as 2+, 3+, or 4+, the latter being also designated as the MID, or minimum inhibitory dose.

Observing these criteria, a preliminary toxicity test is conducted on normal embryonic chick tissue. This toxicity test is followed by two tests on the tumor and normal mouse tissues. *At least* these two tests on the tumors are required in order to cover adequately essential concentrations of the substances being tested, and also to give some assurance of repeatability.

Data typical of tests on the compounds included in this report may be seen in TABLE 2. This table shows the mgm./ml. and comparative millimolar (mM)/ml. concentrations of the four soluble substances. It is possible from the data in this test to conclude that both tumors are affected at the same level by the dimethylaminopurine, that the 8110 tumor is preferentially affected by the carboxamide, that both tumors are affected by the same concentrations of triethylenethiophosphoramide, and that neither tumor is affected by the O-Methyl-L-Tyrosine.

TABLE 2

DATA RELATING TO THE *First* TEST OF THE FOUR *Soluble* SUBSTANCES ON THE MAMMARY 72J AND BRAIN #8110 MOUSE TUMORS

Compound	Concentration		Tumor 8110	LID	Rating	Tumor mammary 72Js	LID	Rating	Comments
	mgm./ml.	mM/ml.							
1. 6-dimethylamino-9-[3′-(L-phenylalanylamino)-β-D-ribofuranosyl]purine	0	0	0			0			8110 and J affected at same level
	.00002	.000045	0			0			
	.0001	.000226	0			0			
	.0005	.00113	0			0			
	.0025	.00565	1–2+	<X	1	2+	<X	1	
	.0125	.02834	4+			4+			
	.0625	.14172	4+			4+			
	.3125	.70862	4+			4+			
2- N⁵-benzyl-2-pyrrolidone-5-carboxamide	1.0	4.58	0			0			8110 most sensitive
	2.0	9.17	0			0			
	4.0	18.35	2–3+	<X	3	0			
	8.0	36.69	4+			4+	<X	3	
3. N,N′,N″-triethylenethiophosphoramide	.05	.34	0			0			8110 and J no LID possibly both affected at same level
	.15	1.03	3–4+	<X	2	4+	<X	2	
	.45	3.10	4+			4+			
	1.35	9.3	4+			4+			
	4.05	27.93	4+			4+			
4. O-methyl-L-tyrosine	.5	2.564	0			0			Neither affected
	1.0	5.128	0			0			
	2.0	10.256	0			0			
	4.0	20.512	0			0			
	8.0	41.025	3–4+	<X	4	0		4	

The two adjacent columns indicate the level at which the designation LID was ascribed and also a rating of the relative toxicity of these four substances with one another.

This test was repeated on the same tumors but with minor adjustments of the concentrations of the substances (TABLE 3). This readjustment of concentrations is most evident for the triethylenethiophosphoramide. Owing to the high concentrations that were used in the first experiment, a graded response was not achieved. In this repeat experiment, accordingly, a closer adjustment of concentrations allowed for the identification of a much more complete spectrum of activity. However, it is obvious that, in general, the test provides data for the same conclusions as the first test.

Included on this chart are the effects of the substances on the normal embryonic mouse skin. In no instance was there a selective effect on the tumor cells over the normal cells.

It is interesting to note that not only does the same relationship of toxicity rating between the two tests on the tumor cells remain identical, but that the normal mouse skin shows the same relationship of toxicity.

Another series of tests, similar in all technical aspects to the previous experiments, was conducted on four relatively insoluble compounds. A test on this factor of solubility is necessary in view of the fact that a large number of potentially interesting compounds for screening are insoluble.

The combined data from our several 48-hour tests of these materials on normal and tumor cells (TABLE 4) provided us with what we felt was sufficient evidence to conclude that, in general, no selective effect for tumor cells over normal cells was effected by these substances.

Considered individually, the malonamide and piperazine compounds are evaluated as having no effect on the tumors in view of the very high concentrations required to induce inhibition. At these very high levels, in all probability unusable clinically, there is a definite effect on the cells of the 8110 tumor. The thiophosphoramide affects both tumors at approximately the same concentrations, while the mercaptopurine shows a definite preference for the 8110 tumor cells.

It was the primary intent of this study to compare the effects of various substances on tumor cells cultivated *in vitro* in order to compare the conclusions drawn from such a test with those arrived at by *in vivo* studies. In TABLE 5, the evaluations sponsored by the two methods are compared. We feel that in view of the very close correlation of these two methods, the few relatively minor discrepancies would be well within the range of many biological systems.

The data accumulated from these few tests alone provide the basis for numerous inferences regarding the effects of compounds on normal and tumor cells. For the time being, however, we prefer to relate our conclusions only to these eight compounds and not to any broad statement which would specify this result as a valid test for any and all chemical substances. A modifier of considerable importance, one on which our restricted conclusion is based, is the fact that with this technique, chemicals which might be modified or changed to an effective form in the animal system, or which might act on some enzyme

TABLE 3

DATA RELATING TO THE *Second* TEST OF THE FOUR *Soluble* SUBSTANCES ON THE MAMMARY 72J AND BRAIN #8110 MOUSE TUMORS

Compound	Concentration		Embryo mouse skin	LID	Rating	Tumor 8110	LID	Rating	Tumor mammary 72 J_8	LID	Rating	Comments
	mgm./ml.	mM/ml.										
1. 6-dimethylamino-9-[3′-(L-phenylalanylamino)-β-D-ribofuranosyl]purine	0	0	0	×	1	0	×	1	0	×	1	8110 and J affected at same level
	.0005	.00113	0			0			0			
	.0015	.00334	0			±			±			
	.0045	.010204	3+			3+			31			
	.0135	.03061	4+			4+			4+			
	.0405	.09184	4+			4+			4+			
2. N⁵-benzyl-2-pyrrolidone-5-carboxamide	2.0	9.17	0	×	3	0	×	3	0	×	3	8110 most sensitive
	3.0	13.76	0			1+			0			
	4.5	20.64	3+			3–4+			+?			
	6.75	30.96	4+			4+			4+			
	10.125	46.44	4+			4+			4+			
3. N,N′,N″-triethylene-thiophosphoramide	.038	.26	0	×	2	0	×	2	0	×	2	8110 and J possibly both affected at same level
	.057	.393	1+			±			0			
	.086	.593	2+			2+			1+			
	.129	.890	3+			2+			4+			
	.194	1.34	4+			3+			4+			
	.291	2.0	4+			4+			4+			
	.437	3.01	4+			4+			4+			
	.656	4.52	4+			4+			4+			
4. O-methyl-L-tyrosine	2.0	10.256	0	×	4	0	×	4	0			Neither affected
	3.0	15.384	1+			1+			0			
	4.5	23.076	2+			1+			0			
	6.75	34.615	3–4+			3+			0			
	10.125	51.923	4+			4+			3–4+	×	4	

TABLE 4

COMBINED DATA FROM THE *First* AND *Second* TESTS OF THE FOUR *Insoluble* SUBSTANCES ON THE MAMMARY 72J AND BRAIN #8110 MOUSE TUMORS

Compound	Concentration		Embryo mouse skin	LID	Rating	Tumor 8110	LID	Rating	Tumor mammary $72J_{10}$	LID	Rating	Comments
	mgm./ml.	mM/ml.										
5. N,N′-di-p-chlorobenzyl malonamide	0	0	0			0			0			Neither affected
	7.5	21.37	0			0			0			
	11.25	32.05	0			±	×	4	0			
	16.87	48.06	±?	×	4	+?			0			
6. 1-carbamyl-4-O-chloro-benzoyl-piperazine	0	0	0			0			0			Neither affected
	7.5	28.02	±?	×	3	+?	×	3	0			
	11.25	42.02	±?			0			0			
	16.87	63.02	+			±?			0			
7. N-(3-oxapentamethylene-N′,N″-diethylenethio-phosphoramide	0	0	0			0			0			8110 & J both affected
	0.1	0.429	—			0			0			
	0.125	0.535	0			±?	×	1	0			
	0.15	0.643	—			±			0			
	0.156	0.668	±	×	1	±			0			
	0.195	0.836	1–2+			+			+	×	1	
	0.225	0.964	—			2+			1–2+			
	0.243	1.042	2+			2+			1+			
	0.304	1.303	3+			3+			3+			
	0.337	1.402	—			3+			4+			
	0.380	1.633	4+			3–4+			3–4+			
	0.475	2.036	4+			4+			4+			
	0.505	2.165	—			4+			4+			
8. 6-mercaptopurine	0	0	0			0			0			8110 most sensitive
	1.0	5.882	±	×	2	±	×	2	0			
	2.0	11.764	+			+			0			
	4.0	23.529	+?			+			0			
	8.0	47.059	1–2+			+			0			
	16.0	94.118	2+			1–2+			0			

TABLE 5

A COMPARISON OF THE IN VIVO AND IN VITRO TESTS OF 8 COMPOUNDS ON THE MAMMARY 72J AND BRAIN # 8110 MOUSE TUMORS

Compound	Mouse mammary $72J_8$ tumor		Mouse brain tumor 8110	
	Tissue culture	*In vivo*	Tissue culture	*In vivo*
1. 6-Dimethylamino-9-[3′-L-phenylalanylamino-β-D-ribofuranosyl]-purine	+	+	+	±
2. N^5-benzyl-2-pyrrolidone-5-carboxamide	−	−	+	+
3. N,N′N″-triethylenethiophosphoramide	+	+	+	+
4. O-methyl-L-tyrosine	−	−	−	±?
5. N,N′-Di-p-chlorobenzyl malonamide	−	−	−	−
6. 1-Carbamyl-4-o-chlorobenzoylpiperazine	−	−	−	−
7. N-(3-oxapentamethylene-N′,N″-diethylenethiophosphoramide	+	+	+	+
8. 6-Mercaptopurine	−	±	+	±?

or similar producing system which could affect the tumor would have a limited possibility of being identified by tissue-culture methods.

It is our intention, however, to extend these tests to other chemical substances in order to gain a further evaluation of this technique and an insight into other influencing factors.

Our experience thus far has indicated that various modifications can be safely introduced into this test, which permit a more rapid evaluation. Such are:

(1) A 48-hour exposure of the substances on the cells provides the same conclusions as longer periods of time or data obtained from sampling at different periods of time. We have reason to believe that even 24-hour exposures are sufficiently long.

(2) Once one has gained sufficient familiarity with the tissues and cells being studied, cultures of minced tissues, instead of accurately cut explants serve the same purpose.

By the same token we have become very much aware of several essential points which must be stringently observed if conclusions are not to be significantly altered. These conditions are:

(1) The cell colonies must be morphologically well spread, and the cells must be in good condition. The use of poorly established cells, even though this condition is taken into account in the overall effect of the substance, leads frequently to confused data.

(2) The tumor tissue used in repeat and comparative tests must come from the very same source. The sensitivity of our test has shown that one cannot use, for example, the mammary 72J tumor and necessarily obtain the same results as with the 69J tumor, in spite of the fact that they are so closely related.

(3) Too much emphasis cannot be placed on the accuracy with which compounds are weighed and then diluted for assaying.

(4) With many types of tumors, several cell forms are involved. It is essential that the correct cell form be read.

(5) In order to obtain the essential or clarifying concentrations of the substances being tested, *at least* two tests on the tumor cells must be run for each substance.

(6) The colonies must be stained for accuracy in reading.

Conclusions and Summary

A tissue culture assay of eight compounds on the mouse mammary 72J tumor and the mouse brain tumor 8110 agrees very closely with the results of the *in vivo* tests of these same compounds. In obtaining these results several essential factors must be observed.

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Discussion of the Paper

Doctor Jane C. Wright (*Director, Cancer Research Foundation, New York, N. Y.*): The results of the experiments on animals reported by Doctor Eichorn and his associates parallel our own observations in connection with the treatment of neoplasms in humans.

In studies undertaken at Harlem Hospital with A-Methopterin and triethylene melamine, it was noted that there was a correlation between the effects of the drugs in patients in whose treatment they were employed and the effects of the drugs on tissue cultures from biopsies of these same patients.[1, 2]

Subsequently, in a study of 18 cases involving 11 different types of neoplastic tumors, further significant data on this correlation were developed in the investigation of the effect of a chemotherapeutic agent on human tumors in tissue culture and the effect of the same chemotherapeutic agent used clinically in the patient with advanced neoplastic disease from whom the biopsy for culture was taken.[3]

The following four drugs were used in this study: triethylene melamine, triethylene phosphoramide, puromycin, and the Wright-West dog duodenum serum preparation.

A close correlation was observed in the results in eight cases and a moderate one in ten cases. In no case thus far studied was a marked discrepancy found between the effects on the tissue culture and the effects on the patient.

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THE EFFECT OF HORMONES AND VITAMIN A ON ORGAN CULTURES

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Organ culture is a type of tissue culture in which the explant grows in a compact, differentiated, and usually functional state, unlike the diffusely spreading, unorganised outgrowth of cells which characterises the more usual forms of the technique. The method is applicable to many physiological and pharmacological problems, and in my talk today I shall give a short review of some recent work in which it has been used to study the action of hormones and of a vitamin.

In the body, hormones and vitamins usually set in motion complicated interactions between different tissues and organs which are very difficult to unravel by experiments *in vivo.* An organ culture, however, provides a closed system in which certain elements of the problem can be isolated and investigated under simplified and more readily controlled conditions. I shall first indicate the type of information that can be obtained by this technique, and then describe briefly the chief results that have been achieved by its means during the past three years.

It is often important to know whether a hormone or vitamin produces a certain response by a direct action on the tissue concerned. This information can sometimes be ascertained by growing the tissue in a medium to which the agent has been added. If the result shows that the action is direct, the physiological effect can be seen histologically and the biochemical reactions investigated by means of modern microchemical methods and the use of isotopes. Organ culture can be used not only to study the effect of an agent on an organ but, conversely, to investigate the effect of tissues and organs on the agent, a point which is sometimes overlooked.

I shall first describe shortly the research that has been done on the direct action of hormones on organs *in vitro.* The earliest experiments in this field were made by Gaillard (1942), who demonstrated an effect of sex hormones on explants of the uterus and seminal vesicles of immature mice. He found that in the presence of oestrone they continued to develop, but controls without oestrone rapidly deteriorated. Recently Wolff (1953) has shown that in the chick the male hormone directly determines the regression of the Müllerian ducts. When the ducts were removed from 7-day embryos and cultivated in normal medium, they developed into female ducts, but if testosterone were added, they degenerated, although explants of the Wolffian duct cultivated in the same medium remained unaffected. Wolff and his collaborators (1952) also found that sex hormones exert a direct action *in vitro* on the undifferentiated gonads of the chick; thus when the testicular rudiment was grown in a medium containing the crystalline female hormone, it produced an ovarian type of structure, instead of forming tubules as its control did in normal medium.

The action of cortisone on organ-cultures of lymph nodes has been investigated by Trowell (1953). It is well known that cortisone kills lymphocytes in the lymphoid tissues of the intact animal, but it was uncertain whether the hormone was directly toxic to the cells or acted indirectly. Experiments with ordinary tissue cultures gave very conflicting results which, in Trowell's view, were probably attributable to the difficulty of maintaining lymphocytes alive and healthy *in vitro* for even a few hours. Trowell, however, appears to have solved this problem by means of an ingenious technique for growing organised lymph nodes of the rat in culture; after two days in normal medium these explants contained only 1 to 2 per cent of pycnotic nuclei, but the addition of cortisone at a concentration of 3×10^{-5} M killed at least 45 per cent of the lymphocytes during this period.

Explants of the early limb-skeleton of the chick are very susceptible to the direct action in low concentrations of thyroxine, insulin, and vitamin A, each of which produces a quite different type of histological change in the same material. A curious fact emerged from these experiments, namely that the primordia of the various long-bones do not all react to a given agent in exactly the same way, but each seems to have its own physiological individuality which determines its characteristic response. The response is also affected by the age of the embryo from which the rudiments are taken and the concentration of the agent in the medium.

I shall first describe briefly some experiments which Sir Edward Mellanby, of the National Institute for Medical Research in London, and I have done on the effect of L-thyroxine on embryonic skeletal tissue *in vitro;* this work is not yet complete, so that I can give only a preliminary account of our results. In 1924, Willier found that when pieces of the thyroid from postembryonic fowls were grafted on the chorio-allantoic membranes of 7- to 10-day embryos, the chicks became emaciated and the limb-bones, particularly those of the leg, were abnormally short; no histological data were given. In postembryonic animals, hyperthyroidism stimulates skeletal maturation and, under certain experimental conditions, may also promote growth; under other conditions growth is retarded and the bones are stunted (Silberberg and Silberberg, 1943; Ray, Asling, Simpson, and Evans, 1950).

L-thyroxine produced two effects on the explants *in vitro:* acceleration of differentiation and sometimes of growth and, later, an inhibition of growth, eventually associated with cellular degeneration. Both effects might occur in the same explant, *i.e.* stimulation during the first few days in culture followed by inhibition of growth and regressive changes. The relative duration and intensity of the two effects depended on the dose of thyroxine, on the age of the embryo from which the rudiments were obtained, and on the particular bone-primordium under consideration.

In rudiments explanted at a precartilaginous or mesenchymal stage, doses of 0.01 or 0.156 mgm. per liter produced mainly a stimulatory effect. When the axial mesoderm is dissected out of a 4- to 4½-day limb-bud and cultivated in normal medium, it enlarges to many times its original size, forms joints and the primordia of the various long-bones, and rapidly chondrifies. The next stage in its development, however, namely hypertrophy of the diaphysial cartilage cells associated with periosteal ossification, is either much delayed or fails to appear. We found that the presence of thyroxine stimulated or greatly accelerated this phase of differentiation in the humerus and sometimes in the tibia, but had little or no effect on the femur, radius, and ulna. More prolonged exposure to the higher dose of 0.156 mgm./liter caused some degeneration in the explants.

Another group of experiments was made on the unossified, cartilaginous rudiments from 6-day limb-buds, the individual primordia being grown separately. In normal medium they enlarged to four or five times their original length, the cartilage cells of the shaft hypertrophied, and a layer of periosteal bone was deposited. If 0.15 mgm./liter of L-thyroxine was added to the medium, the differentiation and usually the growth of the radius was accelerated as compared with its control in normal medium, and even after 10 days *in vitro* it showed little degeneration. The differentiation of the humerus and ulna was also stimulated during the first few days, but the growth-rate then declined and, later, necrosis began to appear. On the other hand, the tibia and, to a slightly less degree, the femur were severely affected; there was little if any stimulation during the early stages of culture and the growth-rate soon began to diminish. In sections it was seen that while hypertrophy of the diaphysial chondroblasts and periosteal ossification took place normally, the intercellular partitions of cartilage matrix were much narrower than in the control.

The action of insulin on 6-day embryonic limb-bones is very different from that of thyroxin and has been extensively studied by my colleague, Doctor Chen. The most obvious effect of this hormone in a concentration of 0.16 unit/ml. was a great reduction in the length of the shaft associated with a striking enlargement of the ends; the effect seemed to be an exaggeration of that noted by Landauer (1945) and later by Duraiswami (1950) in chicks treated with insulin *in ovo*. Histologically the changes were almost the reverse of those produced by thyroxine; instead of accelerating differentiation, insulin retarded it, and whereas thyroxine caused the intercellular partitions of the diaphysial cartilage to become abnormally thin, under the influence of insulin they were slightly thickened; the normal staining reactions of the matrix were unaffected by either agent. As with thyroxine, the various primordia responded to insulin in different degrees; the humerus showed the greatest effect, then the tibia and femur, while the radius and ulna were the least affected. Doctor Chen found that the action of insulin was greatly enhanced by omitting embryo extract from the culture medium. Under these conditions an effect could be obtained with a dose of only 0.0016 unit/ml. Mirsky and Broh-Kahn (1949) discovered that the extracts of certain adult organs inactivated insulin, and experiments have shown that this is also true of embryo extract.

Doctor Chen has also tested the pituitary growth-hormone on cultures of this type. The result was negative, which supports the view that *in vivo* the action of the growth-hormone on the skeleton is indirect.

Sir Edward Mellanby and I have studied the action of vitamin A on embryonic skeletal explants (Fell and Mellanby, 1952). In the young animal the vitamin causes rapid resorption of the bone and cartilage, and spontaneous fractures. It was not known whether the effect *in vivo* was direct or indirect, but the vitamin proved to have a severe direct action on skeletal rudiments *in vitro*, which was quite unlike that of either thyroxine or insulin. When 6-day embryonic limb-bones were grown in medium containing 2000 to 3000 I.U. of vitamin A per 100 ml., metachromasia disappeared from the shaft and eventually from the epiphyses, there was a gradual shrinkage of the intercellular material, but the cells remained alive and sometimes divided. Vitamin A, like thyroxine and insulin, did not affect all the primordia to the same extent; the femur was the most susceptible to its action, then the tibia and humerus and lastly the radius and ulna. The younger the tissue, the more marked were the changes produced, and in the 4-day limb-bud the cartilage either failed to develop or began to form and then disintegrated.

Vitamin A had an even more dramatic effect on explants of the well developed limb-bones of mouse foetuses near them. Controls in normal medium enlarged but otherwise remained unaltered throughout the experiment, but the addition of 3000 I.U. vitamin A/100 ml. of medium caused the large terminal cartilages to lose their metachromasia, shrink, and finally disappear, while the stout bony shaft was rapidly resorbed. On the other hand the surrounding soft tissue grew profusely, and after about 10 days all that remained of the explant was a large sheet of amoeboid cells and a few crumbs of skeletal tissue.

I referred to the use of organ culture to study the effect of the organism on the agent. An example of this was provided by some experiments in which we compared the action of vitamin A introduced 'naturally' into the plasma by feeding the donor fowl on a high vitamin A diet, with that of the same concentration of the vitamin added "artificially" to normal plasma (Fell and Mellanby, 1953). Both media produced a similar type of effect, but the "artificially" added vitamin had a more potent and rapid action than that in the plasma from a hypervitaminotic bird. Sir Edward Mellanby found that this difference in effect was correlated with the extractability of the vitamin in the two plasmas. Thus by shaking the "artificial" high A plasma with petrol-ether, nearly all the added vitamin could be recovered, but the "natural" high A plasma had first to be denatured with 50 per cent alcohol before the vitamin could be extracted with petrol-ether.

We have also studied the effect of vitamin A on various embryonic epithelia. It has long been known that when an animal is suffering from vitamin A deficiency, certain of its mucous membranes undergo squamous metaplasia and keratinisation. When the ectoderm from the trunk and limbs of 6- to 7-day chick embryos was cultivated in high A medium, the reverse effect was produced, *i.e.* keratinisation was inhibited and the epithelium differentiated into a ciliated, mucus-secreting membrane; the active beating of the cilia was clearly seen in life (Fell and Mellanby, 1953).

Work done in collaboration with Doctor S. R. Pelc* shows that this metaplastic change is correlated with a striking difference in the uptake of radioactive sulphate (^{35}S). Glucksmann, Howard, and Pelc† have found that when the radio-active sulphate is injected into mice, autoradiographs of the various organs demonstrate an active uptake of ^{35}S in the mucous epithelia and mucous secretion, but very little in the epidermis. Ectodermal explants which had been grown in normal and in +A medium for 10 days were treated for 24 hours with a drop of Tyrode containing ^{35}S in the form of the sulphate ion. In autoradiographs of histological sections, there was intense blackening over the mucus-secreting epithelium of the +A cultures but very little deposit over the squamous, keratinising epithelium of the controls in normal medium; in both series of explants there was considerable uptake by the connective tissue surrounding the epithelial cysts.

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† Unpublished observations.

This transformation was reversible if the explant was transferred to normal medium. At first the goblet cells secreted with increased vigor, but no new mucous cells were produced; instead, the basal cells proliferated to form a squamous epithelium beneath the secretory layer which, not being recruited from the basal elements, degenerated and was shed. Finally the new squamous layer keratinised.

Experiments were made to see whether vitamin A would stimulate the differentiation and secretory activity of mucous membranes such as those of the embryonic gut and nose, but there was no difference between the explants in high A and in normal medium; both differentiated well and secreted profusely.

The action of the vitamin on two other ectoderm-derivatives, namely the retina and the otocyst of early chick embryos, has also been studied. In a concentration of vitamin A which caused mucous metaplasia in the ectoderm of the trunk and limbs, the retina grew as well as, and usually better than the controls without vitamin A, and differentiated normally. The otocyst also differentiated normally in a medium containing the same high concentration of vitamin A, but was much smaller than its control; this apparent retardation of growth may have been a secondary effect attributable to the complete suppression of the cartilaginous capsule by the vitamin.

At the beginning of my talk, I said that organ culture could be applied to the study of hormone- and vitamin-action in three ways: to find whether a given agent has a direct action on a particular tissue; to investigate the physiological nature of such action; and, conversely, to study the effect of tissues and organs on the agent. So far, most of the work that has been done refers to the first type of problem; the remaining two are only just being approached and offer a very promising field of inquiry.

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FURTHER STUDIES ON THE INHIBITION OF THE CYTOPATHOGENIC EFFECT OF POLIOMYELITIS VIRUSES BY ANTIBIOTIC M-8450 IN TISSUE CULTURE

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The material referred to as antibiotic M-8450 is the broth filtrate obtained after the growth of a mold. The mold producing M-8450 is of the penicillium type and is grown in a complex medium on a rotary shaker incubated at 26° C. The filtrate is prepared by passage of the broth through a Buchner filter to remove the mycelia. It has been used in these experiments either in this form or after it has been reconstituted from the freeze-dried state.

The activity of this mold filtrate was first detected about three years ago on a routine screening program against MM and Semliki Forest viruses in mice. These observations have been reported by Powell *et al*,[1] and later studies on the protective effect of M-8450 against MEFl poliomyelitis virus in mice were described by Powell and Culbertson.[2] Similar protective actions in mice against several other neurotropic viruses have been reported to us by workers in other laboratories, while others have reported its failure to protect against certain viruses of this class. In our own laboratories no protection has been afforded to mice challenged with PR8A and FM-1 influenza viruses, meningopneumonitis, rabies, lymphogranuloma venereum, and lymphocytic choriomeningitis viruses as tested by Powell and his co-workers. In addition, Powell has shown that M-8450 was ineffective against tetanus toxin in mice, and McClain has obtained negative results in embryonated eggs against *Rickettsia prowazeki*. Both *in vitro* and *in vivo* testing has failed to demonstrate any antibacterial spectrum for this mold filtrate.

The inhibition of the cytopathogenic effect of examples of the three immunological types of poliomyelitis viruses in tissue culture by antibiotic M-8450 was reported by us recently.[3] The data presented at that time described the protection of cells grown from the immature monkey testis against the damaging effects of poliomyelitis virus by a 24-hour period of pretreatment with M-8450. The results of these previous experiments have been summarized in Table 1. The cultures shown here received 0.1 ml. of a 1:10 dilution of M-8450 24 hours before being inoculated with 0.1 ml. of undiluted virus. As may be seen, the untreated virus controls were destroyed in four days by types I and III virus and in five days by type II. It may also be noted that the treated cultures in each set remained negative through the 12 days of observation. The subcultures made on the fifth day from pools of both the treated and the control cultures revealed the presence of live virus in both sets, although nearly twice as long an incubation period was necessary to demonstrate the virus in those subcultures made from the M-8450 protected cells. The failure in this experiment to subculture YSK virus from treated cells has been successful in other attempts, but it has been consistently more difficult to recover than the type I 787 and type III Leon viruses. It was also reported in this previous publica-

TABLE 1

EFFECT OF 8450 ON VIRUS GROWTH

Virus type	Treatment	Results Days after virus inoculation				Results of 5th day subcultures	
		4th	5th**	6th	12th		
I	Virus controls	++++ ++++	*** ***			++++	3 days
787	24 hr. pre-treated with 8450	0 0 0	0 0 (lost) 0	0 — 0	0 — 0	++++	6 days
II	Virus controls	+ +	+++ +++	+++ +++		++++	5 days
YSK	24 hr. pre-treated with 8450	0 0 0	0 0 0	0 0 0	0 0 0	0	14 days
III	Virus controls	++++ +++	*** ***			++++	4 days
Leon	24 hr. pre-treated with 8450	0 0 0	0 0 0	0 0 0	0 0 0	++++	7 days

* = Culture medium changed, 10-fold dilution made of original drug and virus.
** = All six groups subcultures.
*** = Discarded.
++++ = Complete cell damage; 0 = no damage.

tion that cells treated with M-8450 10 minutes before or after virus inoculation or simultaneously with it, were not protected from the virus. This observation created doubt that M-8450 acted directly on the virus, and this doubt was further substantiated by experiments which demonstrated that 24-hour incubation of virus with M-8450 did not impair the infectivity of the virus as revealed by subsequent inoculation into cultures.

The method of cultivation of cells employed in these studies has been described in detail elsewhere.[3] The cells obtained from the immature monkey testicle were primarily fibroblastlike, although patches of epithelium were frequently seen. Extensive growth of cells on the glass wall of the roller tube was obtained with a rich culture medium before cultures were transferred to virus experiments. Various types of media have been used during the phase of virus growth, and these types will be described as they occur in the text.

The viruses employed consisted of the fluids from previous tissue culture passages. The 787 and Leon viruses were made available to us by Doctor John F. Enders, the YSK by Doctor Jerome T. Syverton. The western equine encephalitis virus was very kindly supplied by Doctor Seymour Levine.

The method of treatment with M-8450 described previously, namely the addition of 0.1 ml. of a 1:10 dilution to the cultures 24 hours before virus inoculation, has been the method most frequently employed, although other schedules and dosages have been successful. It has been determined, for example, that the 0.1 ml. of a 1:10 dilution can be given at least as long as eight days before challenge with 787 virus, the cells remaining protected. The minimum period of pretreatment with the 0.1 ml. of M-8450 contained in the culture medium has not been determined, but when cells were treated directly with 0.5 ml. of a 1:10 dilution without further dilution of medium, a period of five hours was found necessary to provide complete protection against 787 virus. The protection so obtained could not be removed by extensive rinsing with balanced salt solution just prior to virus inoculation.

In addition to the three viruses previously studied in the monkey testicular cultures, other viruses and other cell systems have been observed. Lymphogranuloma venereum has been grown in monkey testicle cells, in Earle's liver strain, and in our LLC-M2 strain, which was isolated from embryonic mouse skin, but protection by M-8450 against this virus could not be demonstrated in any of these cell types. The western equine encephalitis virus was grown in primary cultures of chick embryo cells and in a strain isolated from the skeletal muscle of a day-old chick, but again no protection of these cells against this virus could be obtained by treatment with M-8450. The western equine encephalitis virus could not be grown in cultures of monkey testes; accordingly it was not known at the time whether M-8450 was inactive against this virus or whether it was ineffective in chick cells. Other viruses including MM, Semliki Forest, lymphocytic choriomeningitis, St. Louis encephalitis, and influenza have been grown in one cell strain or another, but since none of these produced the cytopathogenic effect, evaluation of M-8450 treatment was very difficult.

Of prime importance to certain research projects with M-8450 has been the need for a sensitive assay method. The mouse assay, using doubling dilutions of M-8450 against MM virus challenge has rarely picked up activity in dilutions beyond 1:16. Since the 0.1 ml. of a 1:10 dilution of M-8450 used in tissue culture studies has protected cells against high titer undiluted virus, it was thought that perhaps virus inoculations of as little as 100 I.D. might permit greater dilution of M-8450, thus increasing the sensitivity of the assay. This assumption proved to be false, since it was learned by experimentation that a certain dose of M-8450 was required to protect cells, and that this amount of the antibiotic was sufficient to protect against any amount of virus inoculum. Thus the protective effect of M-8450 appeared to be an all-or-none phenomenon. It was interesting to note that the tissue culture assay attempts ran out at about 1:16 approximately, as had the animal assay, although much smaller amounts of these dilutions were used in the tissue culture studies.

As noted previously, infective virus could be subcultured from undamaged treated cultures. This has since been further studied in order to determine what effect the M-8450 treatment had on virus titer. These results have been tabulated in TABLE 2. In this experiment the virus yield was determined from four sets of conditions which included untreated virus controls, M-8450 treated cultures, virus incubated in tubes with medium but without cells to determine the loss during incubation, and in similar tubes without cells, but to which M-8450 had been added. All cultures were inoculated

TABLE 2

THE EFFECT OF M-8450 ON VIRUS MULTIPLICATION IN TISSUE CULTURE

	3 days	Titer of 3d day pool
0.1 ml of straight virus	++++ ++++	10^{-8}
0.1 ml of straight virus + M-8450	0* +	10^{-4}
0.1 ml of straight virus, no cells		10^{-2}
0.1 ml of straight virus + M-8450, no cells		10^{-2}

* This tube cnly titrated.
0 = no cellular damage.
++++ = all cells destroyed.

with 10^7 tissue culture infective doses of 787 virus and were titered three days later, at which time the control cultures were completely destroyed. As may be seen, the controls produced virus to a titer of 10^{-8}, while the M-8450 treated cultures dropped to a titer of 10^{-4}, or a thousand-fold loss from the 10^7 infective doses contained in the original inoculum. The two sets of tubes incubated with virus and culture medium, but without cells, dropped to less than 10^{-2}, thus indicating that the undamaged cells in the M-8450 treated cultures had helped in some way to maintain a portion of the virus. Further work in progress on this aspect of the action of N-8450 is not available for report at this time.

Of considerable concern has been the question of the site of M-8450 activity and the degree of specificity involved. Since all of the previous work had been done in cultures of rapidly growing cells, it was questioned if this state of cell metabolism were necessary to demonstrate the activity of M-8450 and, if it should be necessary, then one might suspect an intracellular site of action with greater specificity than a possible surface absorbtion. This objective was approached through studies of the effect of temperature and medium on the activity of M-8450 in monkey testicular cells against 787 virus. Medium 199 has been observed not to support active growth of the monkey testicular cells, therefore this medium was employed at 37° C to provide an example of a reduced metabolism by an inadequate medium. Our regular tissue culture medium, containing both horse serum and chick embryo extract, was used as an adequate medium but incubated at 4° C. TABLE 3 shows the results of this experiment. One tenth ml. of 1:10 M-8450 was added to the cultures 24 hours before challenge with 1000 1.D. of 787 virus. Cultures incubated at 4° C were held at this level during the 24-hour period of pretreatment and were then moved to 37° C after virus inoculation. As may be seen, the controls showed that the virus was infective both in regular and medium 199, that the M-8450 was active but not toxic, and that 24 hours of incubation at 4° C did not destroy the cells nor impair their ability to support virus growth when returned to 37° C. The experimental cultures revealed that M-8450 was not active or did not combine with the cells in medium 199 at 37° C. or in regular medium at 4° C. The results of this experiment and repetitions of it suggested that cells in a reduced metabolic state brought about either by inadequate medium or by

TABLE 3

THE EFFECT OF AN INADEQUATE MEDIUM ON THE PROTECTIVE ACTION OF M-8450

	3 days	6 days
Reg. med. virus control 1000 I.D.	++ ++ ++	++++ ++++ ++++
Reg. med. M-8450 cell control	0 0 Trace	0 0 Trace
Med. 199 M-8450 cell control	0 0 0	0 0 0
M-199 + M-8450 1000 I.D. virus	++++ ++++ +	++++ ++++ ++++

THE EFFECT OF LOW TEMPERATURE (4°C.) ON THE PROTECTION ACTION OF M-8450

	3 days	6 days
Reg. med. + M-8450 temperature control for cells	0 0 0	0 0 0
Reg. Med. + 1000 I.D. virus temp. control for infectivity	++ ++ ++	++++ ++++ ++++
Reg. med. + M-8450 + 1000 I.D. virus	+ ++ +++	++ +++ +++

0 = no cellular damage.
+++ = all cells destroyed.

low temperature could not be protected by M-8450 against virus damage. The alteration of physical or chemical conditions in the medium which may govern the combination of cells with M-8450 was not excluded.

The effect of medium 199 and other solutions on the protective action of M-8450 was studied further by testing two other sources of medium 199 as well as serum ultra filtrate (ox) and Earle's balanced salt solution. The data obtained from these experiments have been listed in TABLE 4. The virus controls indicated that all these media allowed virus growth, but that medium 199 from source A allowed more rapid growth in three days than the other fluids. This medium 199, from source A, also constantly appeared to be somewhat toxic to the cells on a morphological basis, while the other samples of medium 199, along with the serum ultra filtrate and balanced salt solution appeared to maintain the cells in as good a condition as did the regular medium. The medium 199, from source A, was the same medium in which M-8450 previously failed to demonstrate protection. As may be seen from TABLE 4, this medium 199 again prohibited the demonstration of the inhibitory action of M-8450, while M-8450, incorporated in the other two sources of medium 199, completely protected the cells from virus damage. M-8450 did not completely protect the cells in balanced salt solution and in serum ultra filtrate; small amounts of virus damage were seen in these cultures by the sixth day. These two fluids have produced these same effects repeatedly. The findings in these experiments cast doubt on the thesis that M-8450 acted only on rapidly metabolizing cells, especially in view of the results obtained in balanced salt solution and serum ultra filtrate unless a quantitative effect was considered. Source A medium 199, in which inhibited protection by M-8450 failed to show activity, as mentioned previously, apparently was somewhat toxic to the cells and may have decreased their metabolic activity even more than the salt solution or serum ultra filtrate; the latter media were not toxic and could have been

TABLE 4

THE EFFECT OF ANTIBIOTIC M-8450 AGAINST 787 POLIOMYELITIS VIRUS IN VARIOUS MEDIA AT 37° C.

	Results at 3 days	Results at 6 days
Regular medium virus control	++	++++
Regular medium + M-8450 + virus	0	0
Medium 199 source A virus control	++++	++++
Medium 199 source + M-8450 + virus	++++	++++
Medium 199 source B virus control	++	+++
Medium 199 source B + M-8450 + virus	0	0
Medium 199 source C virus control	++	+++
Medium 199 source C + M-8450 + virus	0	0
Earle's B.S.S. virus control	++	+++
Earle's B.S.S. + M-8450 + virus	0	+
Serum ultra filtrate virus control	++	++++
Serum ultra filtrate + M-8450 + virus	0	Trace

0 = No virus damage
++++ = Complete destruction of culture.

slightly fortified by traces of the regular medium used for initial cell growth. Since complete protection was not obtained in salt solution and serum ultra filtrate, it could be inferred that metabolism of the cells in these media, which are less nutritional than medium 199, was at a lower rate than in the cells fed on a source B and C medium 199, in which complete protection was obtained. It remained, however, of paramount importance to be able to rule out physical or chemical factors which may have been functioning in these fluids to influence the combination of M-8450 and cells, before definite statements could be made on the effect of cell metabolism on the mechanism of the action of M-8450. The complexity of medium 199 has made the problem of clarifying this point an enormous one, especially if the methods of preparation in the three sources were to be scrutinized for differences which might explain the variation of results obtained with the medium from source A as compared to media from sources B and C.

M-8450 has not proved to be an especially toxic substance for tissue cultured cells although an occasional lot, if added to the cultures undiluted in 0.1 ml. quantities, did cause a slight toxic reaction. This effect has not been noted when the 1:10 dilution was used. To rule out the possibility that the action of M-8450 was merely one of general toxicity on the cells, the effect of the 0.1 ml. of a 1:10 dilution on cell growth was studied by quantitative methods. As the primary monkey testicular cells could not be used in making this study, one of our mouse cell strains, LLC-M2, was observed instead. During the first three days the treated cells increased in number in the same order as the controls, but by six days had dropped behind the controls by 13 per cent. Thus during the usual period of observation, the M-8450 caused very little if any impairment of growth, and even though growth inhibition was noted, by six days it seemed doubtful that this result could be construed to explain the complete inhibition of the cytopathogenic effect of large quantities of virus.

In summary, it has been shown that M-8450 will protect monkey testicular cells grown in tissue culture, under some conditions, against large doses of poliomyelitis virus. It has been evident that the action of the antibiotic was directed to the cells, not the virus, and that a certain period of pretreatment, lasting at least five hours and using large doses of M-8450, was necessary to bring about this protective effect. It has also been shown that the union, or reaction between cells and M-8450 persists for at least eight days and that this protective effect could not be rinsed away easily. Although M-8450 did completely protect cells against the virus damage, these protected cells did in some way maintain more virus than similar tubes incubated with medium, but without cells. The protective effect of M-8450 in cultures incubated at 37° C. could not be uniformly demonstrated in several media tested, nor was the activity of this antibiotic detected in a favorable medium when cultures were incubated at low temperature. No activity of M-8450 was demonstrated against western equine encephalitis virus in two different cell types from chicks, but since this virus could not be cultivated in monkey testicular cells, where the antibiotic was known to be effective, and since poliomyelitis viruses could not be grown in the chick cells, it was impossible to determine whether it was the cell type or the virus which failed to respond. Could a virus be found which would infect both cell types producing a cytopathogenic effect and which M-8450 would protect against in the monkey cells, then this question could be solved. The lymphogranuloma venereum virus which grew in every cell type tested could not be inhibited by M-8450.

The study of M-8450 is being continued on the premise that it does have some effect on the cell which specifically inhibits the cytopathogenic effect of poliomyelitis virus and possibly others, but that it is not a general virus inhibitor. It is felt that, if this premise is true, continued effort may eventually lead to information on the mechanism of virus invasion of the cell and/or virus growth within the cell.

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CELLULAR SENSITIVITY TO DRUG ACTION IN SHORT-TERM TISSUE CULTURES: *IN VITRO* CORRELATIONS WITH SENSITIVITY *IN VIVO*

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In vitro cultivated tissues represent a choice experimental material for research in toxicology. In this paper, I shall report the results obtained from work I have been engaged in during the last 20 years.

I shall deal successively with the following items: (1) How can toxic effects upon cells be evaluated in cultures? (2) Mineral and organic toxic drugs used; (3) differences in sensitivity to drugs of various cellular strains when cultivated *in vitro;* (4) a comparison of sensitivity to drugs *in vitro* and *in vivo;* and (5) How can *in vitro* cultivated tissues acquire a resistance to some toxic drugs?

(1) How Can Toxic Effects upon Cells be Evaluated in Cultures?

Toxic drugs can attack specifically the cytoplasm or the nucleus. On the other hand, they can act on resting cells or on dividing cells. To this last group belong drugs called "mitotic poisons" which in the past have been and, at present, still are the object of important work in the United States and in Europe, expecially at the Belgian University of Liége. Those drugs are, particularly, colchicine, trypaflavin, and beryllium salts.

The drugs I have used show no special effect on the mitotic process. They offset the whole cell. It is believed (R. Fabre) that drugs induce changes in the physicochemical state of protoplasm. They penetrate the cell, modifying colloidal states and permeability. The water content of the cell is changed.

Histologically, the affected cell manifests a reaction expressed always in the same changes of cultural behavior. As Chévremont has observed (1951), cultivated cells lose the power of migration. They often show modifications of their shape. They become round. Their cellular type can be changed. Fibroblasts take the appearance of histiocytes under the action of atropin, as has been described by Spadafina (1933) and by myself (1947). The cytoplasm shows a progressive infiltration by lipid granules. This infiltration may be seen when a hanging-drop explant is not subcultured and its cells are affected by toxic catabolic products.

The origin of such lipids has been discussed in the literature. Do they come from the medium and penetrate the cell because of the increased permeability? Do they result from a degeneration of the cytoplasm with liberation of lipoprotein complexes, in other words, from lipophanerosis?

It seems that the last explanation is the right one, as may be observed when cells are cultivated in a simple saline medium from which an exogenous origin of lipids can be excluded.

Cells showing lipid degeneration do not necessarily die. When carried in a new medium, they assume a normal activity as long as the nuclei remain normal. But, in time, nuclei are injured. They become pyknotic and experience cellular death.

So the signs of the poisoning of a culture are the slowing and stopping of its growth and, at the cellular scale, changing of the shape, lipidic degeneration and pyknosis of the nucleus. Mitosis stops with cessation of cellular migration.

(2) *Mineral and Organic Toxic Drugs Used*

(*a*) *Mineral drugs.* In research conducted in collaboration with C. Sannié (1933), we have systematically explored the action of all metallic ions. To prevent the introduction of factors able to cause disturbances, we have always used a perfectly isotonic culture medium. I shall deal especially with copper, lead, mercury, zinc, cobalt, and nickel salts.

(*b*) *Organic toxic drugs.* I have used potassium cyanide, 2.4-dinitrophenol, and atropin. Dinitrophenol has been used in alkaline solution, atropin as a sulphate. The toxic substance was added in the medium of the subculture, as a solution in water, the concentration of which was such that, after mixing with plasma and embryonic extract, the desirable concentration was obtained.

My assays have been performed on hanging-drop cultures of fibroblasts (from chick embryos' hearts), from liver, from kidney, and from nervous tissues (from chick embryos, human embryos, rat embryos, and newborn rats). Before being submitted to toxic action, the tissues were subcultured three or four times in order to obtain stabilized cultures.

(3) *Differences in Sensitivity to Toxic Drugs of Various Cellular Strains when Cultivated in Vitro*

(*a*) *Metallic salts.* The work I have done in collaboration with Sannié (1933) has allowed us to classify metallic chlorides in four groups: nontoxic or weakly toxic, moderately toxic, toxic, and strongly toxic. The results are expressed in terms of the normality of the solutions used.

(1) In the first group, we put alkaline metals and alkaline earths. However, ammonium, strontium, barium, and manganese salts inhibit the growth of fibroblastic cultures at concentrations between N/120 and N/750.

(2) Moderately toxic metallic chlorides include those which inhibit the growth of fibroblasts at concentrations between N/200 and N/7000. These chlorides, in order of increasing activity, are: iron, lead, aluminum, uranium, beryllium, thorium.

(3) Toxic metallic chlorides are active at concentrations between N/7000 and N/10,000. These chlorides, in order of increasing activity, are: cerium, nickel, cobalt, gold, zinc.

(4) Cadmium and mercury chlorides are extremely toxic, inhibiting growth below N/10,000. Lead, copper, and platinum show a variable toxicity depending on the presence of plasma in the culture medium. Plasma proteins combining with these salts result, in fact, in a protective action; while in a pure saline medium, these chlorides are extremely toxic. For zinc, cobalt, and nickel, the opposite is observed; toxicity is higher in the presence of plasma in the culture medium. Data in this connection are available for fibroblasts in pure culture.

Hepatic cells, kidney cells, and neuroblasts present a peculiar sensitivity toward all metallic chlorides (Verne and Sannié, 1935). I shall consider the most interesting metals: copper, mercury, lead, zinc, cobalt, and nickel. For copper chloride, toxicity toward hepatic cells is manifested at about N/15,000; toward kidney cells at about N/30,000; and toward neuroblasts at about N/10,000, inhibiting the growth of axons.

As regards mercuric chloride, toxicity is manifested at about N/60,000 towards liver, kidney, and numerous tissues. The toxicity of lead chloride is higher towards kidney cells (N/3000) than toward hepatic cells (N/1500) and neuroblasts (N/1000).

We may accordingly conclude that hepatic cells, kidney cells, and neuroblasts, when cultivated *in vitro*, are more sensitive than fibroblasts to the toxic action of some metallic chlorides.

Kidney cells are most sensitive to copper and lead chlorides. Sensitivity to mercury is the same for kidney, liver, and nervous tissue.

Hepatic cells are more sensitive to copper and lead than neuroblasts.

As regards zinc, cobalt, and nickel chlorides, the difference in sensitivity between liver, kidney, and neuroblasts on the one hand and fibroblasts, on the other hand, is small. Sometimes even fibroblasts show a greater sensitivity than the other tissues mentioned.

While fibroblasts are killed by N/4500 solutions, hepatic cells and neuroblasts by N/5000, the limit is N/3000 for kidney cells.

We have noted a remarkable fact: fibroblasts in kidney and liver cultures are more sensitive to toxic drugs than plain fibroblasts from heart cultures. Their sensitivity seems to lie between that of these fibroblasts and that of epithelial cells.

The differences described may be attributable to a variability in the sensitivity of the various fibroblastic strains, depending on their source. It might also be supposed that kidney or liver fibroblasts become more sensitive following injury of the epithelial components of the culture. We think that liver and kidney can be ascribed a toxicity coefficient when studied in hanging-drop cultures. This coefficient can be expressed as the ratio of the concentration of the metallic ion toxic for embryonic heart fibroblasts to the concentration toxic for cultures from organs.

In the case of copper chloride, this ratio is 3:3 for liver cells and 6:6 for kidney cells; in the case of lead chloride it is 5 for liver cells and 10 for kidney; as regards mercury chloride, it is 2 for liver and kidney. We have found that sensitivity toward toxic minerals is the same for cultivated cells, irrespective of their origin, whether these cells are of embryonic chicken tissue, embryonic or newborn rat tissue, or of human embryonic tissue.

(*b*) *Organic toxic drugs.* (*1*) *Potassium Cyanide.* In hanging-drop cultures, cells are killed in a few minutes, when the concentration reaches 1/2000, according to Olivo (1922) and Laser (1933), a finding I was able to confirm.

(*2*) *2.4 Dinitrophenol.* The toxic concentration of this compound is the same for fibroblasts of liver and kidney cells, calculated at about 1/30,000 in medium. A much weaker concentration is required for nervous tissue at about 1/100,000.

(*3*) *Atropin* (*as sulphate*). The toxic concentration of atropin is calculated at about 1/1500 for fibroblasts, for kidney and liver cells, and for neuroblasts.

A remarkable fact is that its toxicity is the same for cultures coming from chicken, rat, or man, although there are marked differences in sensitivity *in vivo*, as we shall see later. The origin of plasma or of embryonic extract is not involved, since the same results are obtained with plasma or extract from chicken, rat, or man.

(4) *A Comparison of Sensitivity to Toxic Drugs* in Vitro *and* in Vivo

It is interesting to compare the toxicity of various drugs *in vitro* to the toxicity of the same drugs on whole organisms. To evaluate this toxicity, I have used data given by R. Fabre (*Traité de Toxicologie*).

Mineral Drugs. For mercuric chloride, which is the most toxic, the lethal dose for man is reached for a concentration in the organism at between 1/200,000 and 1/240,000. In hanging-drop cultures, fibroblasts are killed when concentration reaches N/30,000, that is, at about 1/110,000. Sensitivity is therefore plainly less for fibroblasts *in vitro*. But for kidney and liver cells and for neuroblasts, the lethal dose is approximately at N/60,000; that is, about 1/220,000. It is about the same as for the whole organism.

As regards copper and lead salts, these substances, as I have already said, form compounds with culture medium proteids that are more or less soluble, so that their toxicity is then weaker than in the case of cultures in a pure saline medium.

This protective action of proteids is certainly more pronounced in the organism, so it is not surprising that some cells cultivated *in vitro* show a greater sensitivity to copper and lead chlorides than does the whole organism.

The effects of copper salts on animals, including man, has raised much debate. Death from such toxicity seems attributable to poisoning of the nervous system, causing cardiac and respiratory paralysis. But before the nervous cells are affected, other cells are injured, including those of the liver and kidney, and we have seen that, in hanging-drop cultures, the cells of these glands are more sensitive than are neuroblasts to copper chloride.

As regards lead salts, toxicologists admit that acute mortal accidents are experienced by the organism when the concentration reaches 1/3000. Neuroblasts in culture die at that concentration. But if fibroblasts are less sensitive (1/1000), the lethal dose for hepatic cells is at about 1/5000, and for kidney cells, 1/10,000. As regards zinc, cobalt, and nickel chlorides, the toxic effect is stronger on cultivated cells, even on fibroblasts than on whole organisms.

Organic Toxic Drugs. (*a*) *Potassium Cyanide.* Cultivated cells show toward this toxic compound a much greater resistance than whole animals. This resistance is inversely proportional to the age of the animal. As I have said, the toxic concentration for cultures is at about 1/2000, while for animals a 1/30,000 concentration is lethal.

Laser (1933) has noticed that the cyanide concentration tolerated by cultivated cells totally inhibits respiration. This fact seems to be connected with the power of cultivated cells to live in an anaerobic medium, thanks to their glycolysis.

(*b*) *2.4 Dinitrophenol.* On whole animals, the toxic effect of 2.4 dinitrophenol is at about 1/100,000. It is the same on cultivated neuroblasts. On other cultivated cells, the toxic effect is calculated at about 1/30,000, which is much weaker.

(*c*) *Atropin.* It is known that the toxicity of atropin compounds varies very much according to the animals affected. The limit of a tolerated dose is .75 gm. per kg. weight in a chicken; .70 gm. in a rat; and only .002 gm. in a man.

I have seen, on the other hand, that sensitivity toward atropin of cultivated cells was the same in any animal (chicken, rat, or man). This sensitivity is weak, since a concentration of 1/7500 would be needed to inhibit completely the activity of a culture from any such animal.

Fibroblasts, kidney cells, and neuroblasts have the same sensitivity.

The high sensitivity of the human organism as a whole toward atropin is not observed in cultivated human tissues.

Neither blood plasmas nor embryonic extracts are able to modify the atropin toxicity.

From these data, it may be concluded that *in vitro* cultivated cells show, towards toxic drugs, a sensitivity which often differs from that of whole organisms, the sensitivity being smaller or greater. It may be concluded that, for every drug, the various tissues have peculiar and specific sensitivities.

Fibroblasts show the higher resistance. Kidney cells, hepatic cells, and neuroblasts behave in a variety of ways toward toxic drugs.

(5) *Acquired Resistance of Cultivated Cells to Toxic Drugs*

Mithridatism, the well-known acquired resistance of organisms to some toxic drugs, may be observed also in cells cultivated in a hanging-drop medium. This resistance has been studied as regards copper sulphate by Wilson (1922), who reported that fibroblasts cultivated for 48 hours in a medium containing a 1/100,000 concentration of this salt can afterwards live 42 minutes in a medium containing a concentration of 1/5000, which kills most untreated cells in 26 minutes.

In the case of copper chloride, I have noticed that after having been cultivated in a medium at 1/100,000, fibroblasts grow and divide in a medium in which concentration is at 1/22,500, which is toxic for nonresistant fibroblasts.

Dinitrophenol. With weak concentrations of this compound a rapidly acquired resistance can be obtained. Fibroblasts cultivated for 48 hours in a medium where the drug concentration is 1/90,000 can be subcultured in a medium where the concentration is 1/30,000. Growth proceeds in the same way as in control normal cultures.

Nervous tissue, after 48 hours of cultivation in a medium at 1/160,000, tolerates a concentration of 1/120,000, at which it is normally killed.

Atropin. It is also very easy to obtain resistance with atropin sulphate after 48 hours of cultivation in a medium in which the concentration is 1/3000. Growth is practically normal when fibroblasts are subcultured in a medium where the concentration is 1/5000, irrespective of the kind of plasma used.

The acquired resistance to some toxic drugs of tissues cultivated *in vitro* shows that the phenomenon is essentially a cellular one. However, the mechanism of the phenomenon is not yet very clear. Acquired resistance may be attributable to an acquired hyposensitivity and may be related to a decrease in reactivity of the concerned cells, or it may be caused by a decreased amount of the toxic drug reacting against the cell. This effect in turn may be attributed to a decrease in permeability, preventing penetration of the drug into the cell. Another possibility would be destruction of the drug by a chemical reaction in the cytoplasm.

I have noticed that resistance to the drug persists even after cell division. However, the daughter cells exhibit the phenomenon of resistance for only a relatively short time. Thus, after four subcultures, resistance to dinitrophenol as well as to atropin diminishes and disappears.

The toxic effects are apparent. Vollmar and Li (1940) have obtained similar results adapting fibroblast cultures to salvarsan. I think that this adaptability is related to a cytoplasmic alteration which, however, is able to persist only through a small number of cell divisions. Each mitosis tends to bring the cytoplasm back to its genotypic constitution.

Such data are to be compared with those obtained by Jollos (1921) on *Paramecium* cultures. Resistance towards arsenious acid displayed by such infusoria persists a few weeks and then disappears.

In my opinion, as regards both infusoria and *in vitro* cultivated cells, the change is a cytoplasmic one not inherent in the genotype. After withdrawal of the inducing factor, the change persists for some time and then gradually disappears owing to the synthesis of protoplasma in new cells, in the same way that an adaptive factor gradually disappears through dilution.

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Discussion of the Paper

Doctor Raymond L. Cahen (*Bristol-Myers Company, Hillside, N. J.*): The exhaustive study of Doctor Jean Verne provides extremely valuable data on the pharmacological activity of drugs and makes it possible to solve the difficult problem of their site of action. Concerning the terminology, I am wondering whether Doctor Verne has not been investigating the "*tolerance*" or "*resistance*" to some toxics (atropine, dinitrophenol) rather than the "addiction," a term which is referred to by Tatum and Seevers as the psychosomatic complex noted with morphine and related substances. "Addiction," besides "tolerance," concerns the physical phenomenon "habituation" and the physical phenomenon "dependence."

TISSUE CULTURE OF HUMAN SKIN AND CHICK SPLEEN AS A METHOD FOR EVALUATING THE PRIMARY IRRITANT PRODUCING CAPACITY OF TOPICAL MEDICAMENTS; CORRELATION OF RESULTS WITH CLINICAL OBSERVATIONS*

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One of the essential facets in the study of therapeutic agents, which are used for topical application to skin and mucous membranes, is the determination of their toxic or primary irritant potentialities. Various methods which are useful in calculating the safe topical dose of a given drug include the performance of so-called prophetic patch tests, the accumulation of clinical experience, and the determination of the comparative irritant effect on the conjunctivae and/or cornea of the rabbit's eye. All of these procedures are utilized by pharmaceutical houses in evaluating new medicaments before they are released for general use.

The purpose of this paper is to present our findings in regard to the preliminary evaluation of another possible method for estimating the potential irritant properties of topical medicaments, namely the use of cultures of human skin and embryonic chick spleen *in vitro*. In other words, do the comparative toxic effects of certain topical therapeutic agents on explants of human epithelium and chick spleen, cultivated in tissue culture preparations, correlate with clinical experience, patch tests, and the rabbit's eye conjunctival and corneal tests in respect to their primary irritant producing potentialities?

The use of tissue-culture methods in the study of the toxic effect of drugs on explants of various tissues, grown in tissue culture, has been reviewed recently by Pomerat.[1] Studies which have been reported by Pomerat[2, 3] and his group have demonstrated that the outgrowth of human epithelium in tissue culture is uniform and, in general, quite constant, except for considerable variation in growth potential which exists in explants obtained from different individuals.

All the drug toxicity tests† on human skin summarized[4, 5, 6] in this paper were done on skin which was taken from patients during skin grafting (split thickness so that only the epithelium and a few fibroblasts were included) procedures.

The skin was cultured by the use of the method established by Lewis, Pomerat, and Ezell[2], the basic steps of which are as follows:

(1) Fragments, approximately 2 mm. square, are cut for explanting.

(2) These explants are embedded in a clot composed of one drop of cockerol plasma (1000 gamma per ml. of medium of penicillin is added to prevent bacterial contamination) and one drop of embryonic extract containing the test substance to be tested.

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† The drug toxicity tests reported here were done as part of a joint project with Doctor Charles M. Pomerat at the University of Texas School of Medicine; other collaborators in the performance of these studies were Doctor Eva T. Everett and Mrs. Patricia Johnson. Many of the "cup" patch test experiments were done by Doctor J. Fred Mullins.

(3) After the clot has set firmly, four drops of malignant ascitic fluid are added.

(4) These clots are made on cover glasses, and after the ascitic fluid is added, they are mounted on hanging-drop slides, sealed with paraffin, and incubated at 37° C. for eight days. Each day, the cultures are examined microscopically and the amount of outgrowth is recorded.

Cultures containing increasing concentrations of the drugs to be tested were prepared. The smallest quantity required to effect the first evidence of injury to the epithelial cells as compared with untreated control cultures was determined and designated as the least injurious dose, or LID. The smallest quantity required to effect total inhibition of outgrowth was considered as the minimal inhibitory dose, or MID. These readings were determined by estimating the extent of migration of epithelial cells as measured under the low-power field of the microscope.

The toxic effect of almost all of the drugs which were included in this study were tested in the same manner on explants of spleen from 18-day chick embryos; in these experiments, ascitic fluid was not added to the clot, and the final results were recorded at the end of 24 hours rather than at the end of eight days as in the case of the human skin explant experiments.

Results

The minimal inhibitory dose (MID) and least injurious dose (LID) expressed in gamma per ml. culture media for 14 drugs or antibiotics which have been used for the topical treatment of skin diseases, three chemicals which are not therapeutic agents (2,4 dinitro-chlorobenzene, nickel chloride, and barium chloride), and one experimental antibiotic (Endomycin) are presented in Tables 1, 2, and 3. We have had sufficient clinical experience with four of these drugs and four antibiotics (listed in Table 1) to justify an analysis of their comparative irritant-producing capacity when applied to intact and diseased skin and to mucous membranes. For this reason, these eight topical medicaments were selected for discussion regarding the correlation of the *in vitro* results and clinical experience.

Detailed analysis of the toxicity data included in Table 1 reveals that gramicidin, bichloride of mercury, Merthiolate, and Furacin are very toxic for epithelial cells and embryonic chick spleen explants, whereas the three antibiotics are essentially nontoxic. The toxicity of the other three antibiotics compares rather closely with the toxicity of sodium chloride, which has an MID, for skin, of 3900 to 7800 and, for chick spleen, of 12,500 to 25,000. The other drug (Quotane) is distinctly less toxic than gramicidin, the mercurials, and Furacin; it is many times more toxic than the three antibiotics. There is considerable variation in the range of toxicity among the various drugs and antibiotics which are included in this group. Also, there is a significant difference in regard to the range of toxicity when the results on human skin are compared with those obtained on the chick-spleen explants. For example, with gramicidin, the entire range for human skin is 0.1 μg. to 1 μg., whereas the range for chick spleen with the same antibiotic was found to be 5 to more than 500 μg. In

Table 1

Toxicity in Gamma per Ml. of Culture Medium of Various Topical Medicaments for Explants of Human Skin and Chick Spleen (for Comparison with Clinical Experience)

Substance	Tissue	LID	MID
Gramicidin	Human skin	0.1–0.2	0.5–1
	Chick spleen	5–10	500–*†
Bichloride of mercury	Human skin	1–2.5	6.25–12.5
	Chick spleen	1.6–3.2	50–100
Merthiolate	Human skin	0.8–1.7	8–17
	Chick spleen	0.125–0.25	25–50
Furacin	Human skin	0.5–1	10–20
	Chick spleen	12–25	100–*†
Quotane	Human skin	2–3	125–250
	Chick spleen	10–20	625–1250
Penicillin-G sodium	Human skin	1250–2500	5000–10,000
	Chick spleen	940–1875	75,000–150,000
Bacitracin	Human skin	830–1655	5000–10,000
	Chick spleen	625–1250	20,000–*‡
Neomycin sulphate	Human skin	830–1015	4165–6250
	Chick spleen	780–1560	250,000–500,000

* Higher concentrations could not be obtained owing to low solubility.
† Marked inhibition, close to complete inhibition.
‡ Partial inhibition.

all instances, the total range of toxicity was much more marked in the case of the chick-spleen cultures. However, there is also considerable variation among the drugs in regard to their range of toxicity for the epithelial explants; with Quotane, concentrations as low as 2 μg. per ml. of culture medium affected slight but significant inhibition, whereas concentrations of the order of magnitude of 125 to 250 μg. were necessary to cause complete inhibition; with bichloride of mercury, the range was only 1 to 12.5 μg. If only the MID is

Table 2

Toxicity in Gamma per Ml. of Culture Medium of Various Drugs and Chemicals for Explants of Human Skin and Chick Spleen (for Comparison with Closed "Cup" Patch Tests)

Substance	Tissue	LID	MID
Bichloride of mercury	Human skin	1–2.5	6.25–12.5
	Chick spleen	1.6–3.2	50–100
2,4 dinitro-chlorobenzene	Human skin	1.25–2.50	12.5–25
	Chick spleen	not done	not done
Thephorin	Human skin	2–2.5	25–30
	Chick spleen	60–125	1000–1250
Nupercaine	Human skin	1.25–2.5	25–30
	Chick spleen	30–60	156–625
Quotaine	Human skin	2–3	125–250
	Chick spleen	10–30	625–1250
Nickel chloride	Human skin	–6	50–100
	Chick spleen	–1250	7500–15,000
Resorcinol	Human skin	2.5–3	500–1000
	Chick spleen	160–320	2500–5000
Barium chloride	Human skin	125–250	1000–5000
	Chick spleen	–5000	25,000–50,000

TABLE 3

TOXICITY IN GAMMA PER ML. OF CULTURE MEDIUM OF VARIOUS ANTIBIOTICS FOR EXPLANTS OF HUMAN SKIN AND CHICK SPLEEN (FOR COMPARISON WITH COMPARATIVE EFFECT ON CONJUNCTIVAE AND/OR CORNEA OF RABBIT'S EYE)

Antibiotic	Tissue	LID	MID
Endomycin	Human skin	50–100	250–500
	Chick spleen	100–200	2500–5000
Aureomycin HCl & sod. glycinate	Human skin	105–210	830–1250
	Chick spleen	155–310	5000–*†
Polymyxin B sulphate	Human skin	105–210	830–1665
	Chick spleen	310–625	10,000–*†
Streptomycin	Human skin	210–415	2220–6665
	Chick spleen	155–310	300,000–*†
Bacitracin	Human skin	830–1665	5000–10,000
	Chick spleen	625–1250	20,000–*‡
Neomycin sulphate	Human skin	830–1015	4165–6250
	Chick spleen	780–1560	250,000–500,000

* Higher concentrations could not be obtained owing to low solubility.
† Marked inhibition, close to complete inhibition.
‡ Partial inhibition.

considered, the order of decreasing toxicity for the skin explants would be as follows: gramicidin, bichloride of mercury, Merthiolate, Furacin, Quotane, neomycin sulphate, bacitracin, and penicillin-G sodium; the order of decreasing toxicity of these drugs for embryonic chick spleen cultures would be as follows: Merthiolate, bichloride of mercury, Furacin, gramicidin, Quotane, bacitracin, penicillin-G sodium, and neomycin sulphate.

In Table 2, we have summarized similar data for various drugs and chemicals which were selected for closed "cup" patch tests, designed in such a manner that the minimal average irritation producing concentration of the eight drugs or chemicals was determined for intact human skin. In this group, we have included two very toxic substances, four of which are somewhat less toxic for human epithelium, another which is comparatively nontoxic and, finally, barium chloride, which is much less toxic than the other seven substances. It is interesting to note that nickel chloride was comparatively toxic for human epithelium, but comparatively nontoxic for chick spleen; indeed, there was more discrepancy in these results (human skin compared with embryonic chick spleen) with nickel chloride than was the case with any other drug, antibiotic, or chemical which we tested.

Data regarding the irritant effect of six antibiotics on the conjunctivae and/or cornea of the rabbit's eye are available, and it is for this reason that the *in vitro* toxicity results of these six antibiotics are summarized in Table 3. Streptomycin and neomycin have an unusually wide range of toxicity for chick-spleen explants. Endomycin was distinctly more toxic than the other five antibiotics, and neomycin sulphate, bacitracin, and streptomycin were decidedly less toxic than polymyxin B sulphate and the Aureomycin preparations.

Correlation of Tissue Culture Results with Clinical Experience

The four very toxic drugs, gramicidin, bichloride of mercury, Merthiolate, and Furacin, are used as topical medicaments in very low concentrations;

specifically for Merthiolate, the recommended concentration is 0.1 per cent, for mercury bichloride 0.1 per cent, for Furacin 0.2 per cent, and for gramicidin 0.025 per cent. Ointments intended for application to the conjunctivae are used in concentrations of not more than 0.02 per cent for Merthiolate and Furacin, and 0.005 per cent in the case of gramicidin. Concentrations of these drugs of the order of 0.3 to 1 per cent result in a significant number of primary irritant reactions, particularly when applied to denuded or eczematized skin.

An ointment or lotion vehicle containing 0.5 per cent Quotane does not cause primary irritant reactions even when applied to denuded or eczematized skin, but the same concentration tends to cause stinging of the conjunctivae, and its use in this manner is contraindicated. When the concentration of Quotane in an ointment vehicle is increased to 1 per cent or more, there is a distinct tendency for the production of primary irritant reactions in some patients.

On the other hand, the three antibiotics, which have a very low order of toxicity for both human epithelium and chick-spleen cultures, also have a very low primary irritant producing capacity when they are applied topically either to eczematized skin or to mucous membranes. It is well known that topical penicillin preparations have a tendency to produce sensitization reactions in a very high percentage of cases, but concentrations of as much as 6 per cent of penicillin may be applied to the conjunctivae without producing primary irritation.

Correlation of Tissue Culture Results with Closed "Cup" Patch Tests

The continuous application of aqueous solutions of the eight substances listed in Table 2 to intact human skin was made possible by strapping glass cups, containing the solutions, to the flexor surface of the forearms of human volunteers. With this technique, it was determined that concentrations of from 0.3 to 1 per cent of bichloride of mercury and 2,4 dinitro-chlorobenzene produced primary irritant reactions in almost all subjects. The primary irritant concentrations of Thephorin, Nupercaine and Quotane, determined in this manner, were from 3 to 5 per cent; similar degrees of primary irritation were produced with 10 to 15 per cent nickel chloride, and with 15 to 20 per cent resorcinol; 20 per cent barium chloride did not produce primary irritation. Comparison of these patch tests results with the toxicity data, as outlined in Table 2, indicates that there is good correlation, except for nickel chloride; in this case, if we consider only the human epithelial results, nickel chloride should be a primary irritant in concentrations of 3 to 5 per cent, but the chick-spleen results would indicate that the primary irritant concentrations might be expected to be of the order of 10 to 15 per cent. It should be noted that, using these closed-patch tests, we could not detect a significant difference between Thephorin, Nupercaine and Quotane, despite the fact that the MID level of Quotane is considerably less for the human epithelial explants than Thephorin and Nupercaine; however, it might be significant that the LID levels of Quotane for epithelium are almost identical with the LID levels of Thephorin and Nupercaine.

Comparison of Irritant Effect on Conjunctivae and/or Cornea of Rabbit's Eye with Tissue Culture Toxicity Results

We do not have our own experimental data for conjunctival and/or corneal tests on the eye of the rabbit, but we do have two sources of information regarding such results. Wiggins[7] has reported that the toxic effects of topical applications of polymyxin B on the corneal epithelium of the rabbit were similar to those of Aureomycin, and that both were more toxic than streptomycin. These findings correlate closely with our tissue-culture toxicity results summarized in Table 3.

Another basis of comparison for Endomycin, bacitracin, and neomycin sulphate is that there is an absence of primary irritation of the conjunctivae of the rabbit's eye with concentrations of 3 per cent bacitracin and 3 per cent neomycin, whereas 1 per cent Endomycin causes marked irritation, and indeed severe chemosis.[8] This effect represents a very significant difference and, of course, it is paralleled by our tissue culture results inasmuch as Endomycin is a comparatively toxic substance, whereas bacitracin and neomycin sulphate have a very low degree of toxicity for both human epithelial and chick-spleen explants.

Discussion

It is apparent that it is feasible and entirely satisfactory to use the patch-test method for detecting gross differences in the primary irritant producing capacity of drugs and chemicals. For example, when this test is applied to the intact skin of only a small number of volunteers, it soon becomes apparent that a comparatively irritant chemical such as bichloride of mercury tends to produce primary irritation of the skin in the majority of cases when the concentration is of the order of 1 to 2 per cent, whereas 0.1 per cent concentration does not cause irritation. Therefore, it may be concluded that the safe topical dose of bichloride of mercury is 0.1 per cent concentration of the chemical in aqueous solution or in an ointment vehicle. In a similar manner, it may be determined that a comparatively nonirritant chemical such as barium chloride is not a primary irritant with concentrations as great as 20 per cent aqueous solution. However, there is no method which makes it possible to discriminate between materials where differences in irritation are slight.

As we have stated in a previous paper,[6] it is desirable to have a test which will "tend to predict borderline irritant effects which may be manifest only under circumstances which are favorable for the development of irritation, such as a breakdown in the skin barrier encountered as the result of maceration due to climatic conditions or partial denudation of the epithelium which results in a loss of the protective keratin layer. These are the conditions which are encountered in clinical practice, and usually topical medicaments are applied to denuded, inflamed, and eczematized skin. It is essential to depend on clinical experience which is accumulated by applying the topical therapeutic agent to the affected sites of patients with various diseases of the skin and mucous membranes. The collection of such data is a very slow process and, indeed, the accurate evaluation of a new drug in this respect may involve the accumulation of experience for as long as several years."

It is for these reasons that we have attempted to utilize this technique as a possible procedure for determining the potential primary irritant effects of old and new drugs when they are applied topically to inflammatory conditions of the skin and mucous membranes. It would appear that the toxicity results which we have reported in this and other papers are reproducible and accurate within comparatively narrow limits of error. Also, the results which we have recorded are in general agreement with those recorded by other workers,[9-25] although the only investigators of this group who employed human skin—rather than other tissues—were Cruickshank and Lowbury.[9] Furthermore, it is apparent that in general the correlation of our toxicity data with the clinical experience, patch tests, conjunctival and corneal tests is quite good, but we have not demonstrated to our satisfaction that we are able to discriminate between relatively slight difference in degree of irritant producing capacities of a drug which, for example, has a MID of 25 to 30 as compared with a drug which has a MID of 125 to 150. Perhaps a variation of toxicity of this degree is not significant, but this inference remains to be proved or disproved.

It is pertinent to comment on the fact that this method is not applicable for drugs which do not tend to be soluble in water. In the case of drugs which are not water-soluble, we have attempted to use various solvents such as acetone, but under these circumstances, the results may be somewhat unreliable. Also, it is well to question the validity of results with drugs which tend to deteriorate in solution, *i.e.*, Aureomycin.

Other facets of this problem which must be studied further include the evaluation of the significance of the wide variation in range of toxicity which is noted in the case of certain drugs and the discrepancy between results obtained with the human epithelial explants and the embryonic chick-spleen tissue. It is possible that the results with embryonic chick tissue are more significant than those recorded for human epithelium.

In conclusion, we have not proved the validity of this method, except as regards the fact that grossly irritant drugs or chemicals are very toxic for both human epithelial cells and chick embryonic spleen when cultivated in tissue-culture preparations, and that comparatively nonirritant drugs have a very low degree of toxicity. We are continuing these studies in an effort to define more accurately the limitations and applicability of this technique as a possible method of "scout" testing new drugs in regard to their primary irritant producing capacity.

Summary

(1) The toxicity of 18 drugs or chemicals were determined for the explants of human skin and embryonic chick spleen in tissue culture.

(2) The relative toxicity of these drugs as determined by the tissue-culture technique are compared with their comparative primary irritant-producing capacity, which is estimated by clinical experience, closed "cup" patch tests, and irritant effect on conjunctivae and cornea of rabbit's eye. In general, there was gross correlation.

(3) The practical application of this procedure for "scout" testing of new therapeutic agents for topical use in respect to potential capacity for causing irritation is discussed.

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Part III. The Action of Chemical Agents on Cell Organoids and Mitosis

NUCLEAR AND CYTOPLASMIC ORGANOIDS IN THE LIVING CELL

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The principle of nonuniformity in protoplasmic units is of basic importance to the study of pharmacologic actions. It is desirable to re-examine some of the older concepts of nucleus and cytoplasm to find whether the component semipermanent structures, defined as organoids, can yield clues to functional problems. Tissue culture is now becoming particularly useful as a tool to study the single cell—although it is noteworthy that the voluminous literature has dealt far more with mass reactions of cells, rather than with internal phenomena inside individual cells. In fact, experimental operations in the cytoplasm or nucleus of a single tissue-culture cell have been remarkably infrequent. It is toward this latter direction that this paper is directed. Particularly its purpose is to present some new facts and interpretations of the metabolic function of the nucleus and the establishment of the division mechanism from the nuclear colloids. Observations and experiments on some other organoids, such as cilia and the nuclear membrane, have been included as they may prove useful in study of special pharmacological problems.

Materials and Methods

Our tissue cultures have been made chiefly from excised fragments of the frog kidney adenocarcinoma. Phase contrast photomicrographs of normal and malignant frog kidney cells are illustrated in FIGURES 1 and 2. An enlarged view of an adenocarcinoma cell undergoing microdissection is shown in FIGURE 3. This tumor has been thoroughly investigated by Lucké (1952), who also introduced the tissue-culture method and transplantation into eyes as techniques for studying these cells. To his scholarly pioneer work we owe much, particularly his basic conclusions that the tumor is transmitted by a virus and that it is characterized by easily recognizable cytoplasmic and intranuclear inclusion bodies. We have found it useful to replace the clot technique of Lucké by a modification of the Earle perforated cellophane† method, combined with the double cover slip method of Maximow. Explants approximately 1 mm.^{3} were excised from firm areas of the tumor, normal kidney, ovary, testis, lung, or urinary bladder, and mounted in 0.05 cc. liquid medium on 18 mm. circular cover slips. Each was covered by a 12 mm. square of sterile cellophane presoaked in medium. One corner of the cellophane square was snipped off as a reference point. Thus cells and fragments that became affixed in different holes might be subsequently identified in daily microscopic studies. For microdissection or photography it was occasionally desirable to remove the

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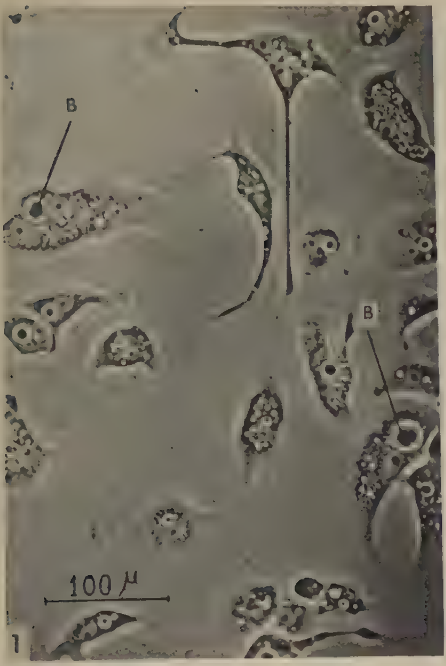
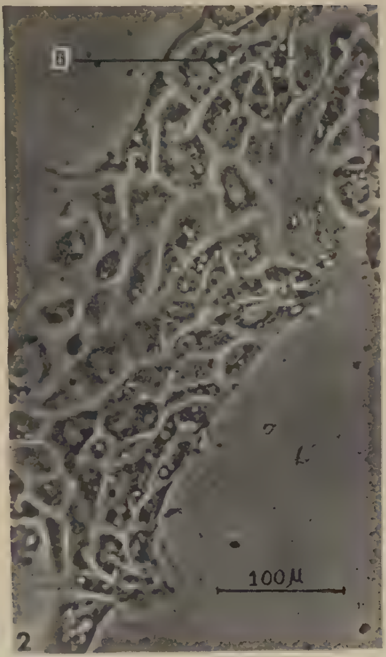
† Doctor Earle kindly supplied DuPont perforated cellophane with the following specifications: average thickness 450 μ, circular holes, Beckley perforation design OA, PT62, average diameter 500 to 600 μ, 17.5 per cent open space.





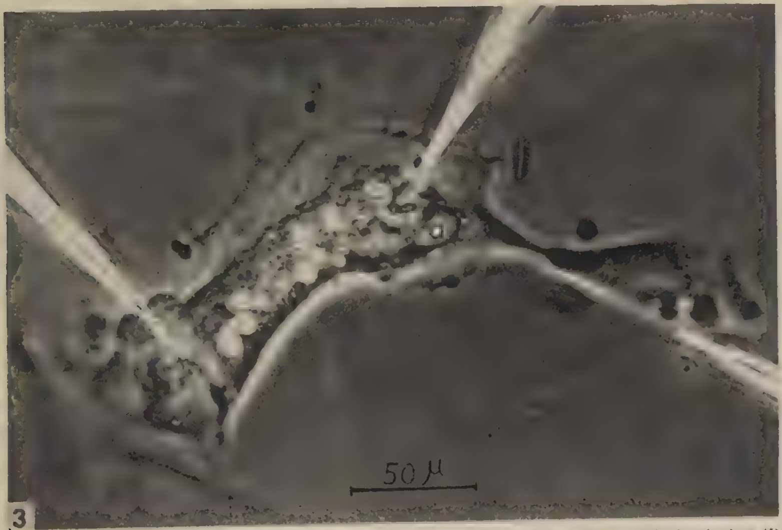


FIGURE 1. *Rana pipiens*. Culture of living normal kidney epithelial cells. Note inclusion bodies (B) and prominent nucleolus in each nucleus. Phase contrast.

FIGURE 2. *Rana pipiens*. Spreading sheet of living kidney adenocarcinoma cells. Note inclusion body (B). Phase contrast.

FIGURE 3. High magnification of a single adenocarcinoma cell undergoing microdissection with three needles. Middle one in nucleolus. Phase contrast.

cellophane entirely, although cultures preferred to remain covered. The following liquid medium, based on commercially prepared ingredients, supported vigorous proliferation of amphibian tissues on the average 2.5 months, some cultures lasting over four months:

Horse serum (frozen)*		2.0 cc.
Chick-embryo extract (fresh-frozen)*		2.0
Fischer's V-614*		2.0
Penicillin solution (20,000 units/cc)		0.05
Amphibian ringer solution (Selas filtered):		3.0
NaCl	0.66 gm.	
KCl	0.014	
$CaCl_2$	0.014	
Glucose	0.10	
II distilled water	300 cc.	

pH of the above medium was 7.6 ± 0.1, as measured with a Beckman glass electrode meter.

It was found that with two washings per week, consisting of 0.5 cc. fresh medium slowly dripped over the inclined cellophane-covered circular cover slip, did not disturb the adherent cells, and maintained satisfactory growth. Cultures grew best, as shown earlier by Lucké at 26° C.

In order to obtain most satisfactory optical results with phase-contrast microscopy, special Pyrex slides with straight sides and flat, ground, and polished bottoms were used.†

Microdissection of cellular organoids, isolation of nucleoli and chromosomes was carried out with a new model electric micromanipulator described by V. Bush, Duryee, and Hastings (1953). Microneedles and the moist chamber with sliding top were modified from standard methods of Chambers and Kopac (1950). Isolated egg nuclei were obtained by dissection and washing techniques described earlier (Duryee 1950).

Cytoplasmic Organoids

Many cytoplasmic components readily observable under the light microscope or by electron microscopy are often confused with those more permanent structures for which the term *organoids* should be reserved. Because they have been so extensively studied and reviewed elsewhere, no detailed attempt will be made to classify or consider them here. One needs only to mention the two great groups or classes of formed bodies and inclusions that will be dealt with by other members of this conference—namely the mitochondrial or chondriome types and the structural organelles, such as myofibrils, cilia, axones, and secretory elements. It is debatable whether structures derived from the pellicle should be included. From a more specialized point of view, bodies such as plastids might belong to the first category, while the endoplasmic reticula, lipid granules, and microsomal fractions could be excluded. In our amphibian cell cultures, only one type of cytoplasmic organoid, the cilia, will be offered for consideration. Historically, the cilia have been useful in studies of pharmacologic effectiveness.

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* Purchased in ampoules from Microbiological Associates, Bethesda, Md.

† Satisfactory slides of this nature are sold under the name *Romicron* by Paul Rosenthal, New York, N. Y.

Ciliary Action. In cultures of some frog adenocarcinomas and on all normal salamander lung and urinary bladder tissues, cilia have developed. It is of interest that peritoneal epithelium, which gives rise to the cilia, can originate either from males or females. This finding appears to contradict the conclusion of Rugh (1951) that cilia are in themselves secondary sex characters. Ciliary activity may start either in a few days in the case of lung tissue, or not until after 6 to 12 weeks in the culture of cancer tissue. Here it may be assumed to have differentiated from residual nephrostome cells, since in cultures of excised normal kidney fragments, cilia may be observed beating in transilluminated nephrostomes.

Of particular interest is the fact that cilia in sterile cultures were exceptionally easy to start and maintain. They are of potential use to pharmacologists as a sign of motility in such cultures. A simple experiment illustrating their vigorous activity and resistance to temperature change is worth reporting. When a crystal of dry ice was touched to the outer cover slip, a sharp wall of advancing ice crystal crept across the microscope field after a few seconds. Ciliary beating was observable at the normal rate (3 to 4 double strokes per second) with slowing just as the ice crystals almost obscured the view. When the dry ice crystal was removed in a few more seconds, the cilia could be seen to have stopped entirely, as the inner serrated wall of ice crystals retreated. From 5 to 60 seconds later, the cilia usually resumed beating, at first slowly and then, in a few more seconds, again at maximum rate. On a given group of ciliated cells in culture such an experiment could be repeated several times before permanent injury was incurred.

Another type of observation, illustrated in FIGURE 4, is significant for the Kinetodesma Theory (*cf.* Metz 1953). As long as the basal granules are connected by visible fibers in longitudinal rows, the cilia beat synchronously at a

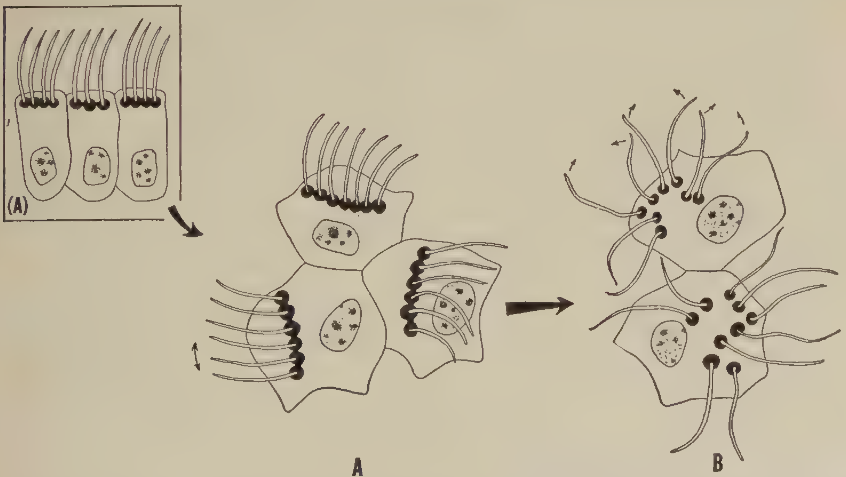


FIGURE 4. Diagram showing dependence of synchronization of cilia on basal granule linkage *in vitro*. Insert at left indicates probable configuration *in vivo*. In A cilia beat synchronously at maximum rate. In B cilia beat at random and usually slower.

maximum rate. However, when granules are separated either spontaneously or with microneedles, the individual cilia beating becomes random and disorganized, slowing to 1 to 2 strokes per second. No recovery from these effects has been observed.

To add to these observations is the minor fact that individual ciliated cells may break off from an epithelial sheet and round up, becoming free-swimming for several days. Cilia distribution on these rounded cells may vary from complete spherical coverage to small tufts of 1, 2, or 3 cilia. An analogy of these "individualized" metazoan cells to spermatozoa is rather obvious, but apparently without special significance. It should not be overlooked, however, that with rapidly advancing techniques in micromanipulation, single free-swimming ciliated cells from a vertebrate adult animal may prove valuable as experimental objects for drug action.

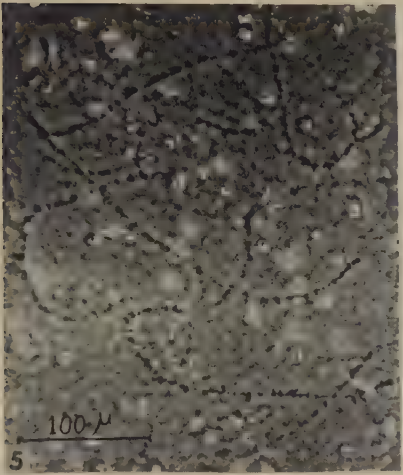
Organization in the Nucleus

New data on this interesting organoid have been accumulated over a period of about 12 years. Only now has it been possible to compare organized features of somatic nuclei, such as are seen in tissue culture, with germ cell nuclei as being studied in transparent eggs and in the isolated condition. As lucidly pointed out by Schrader (1953), understanding of mitosis may well depend on more accurate knowledge of how the spindle is set up from the pre-existing interphase nucleus. Of many possible details, only a few of the more important can be mentioned here:

Chromosomes as organoids are functional during the interphase. Clear evidence on this point has been almost entirely lacking, except for the striking case of the egg nucleus, where chromosomes are known to expand longitudinally and at the same time to grow lateral branches (clusters of loops) which later slough off and are added to the cytoplasm at the time of germinal vesicle breakdown. This principle has been confirmed by other observers, including Guyénot (1953), Dodson (1951), and Gall (1952).

A particularly interesting case of a vertebrate hybrid is shown in FIGURES 5, 6, and 7. Illustrated are isolated, unfixed chromosome pairs from a 10-year-old salamander kindly sent to me by Doctor T. Kawamura of Hiroshima University. The cross described by him in 1950 was *Triturus pyrrhogaster* ♀ by *Triturus ensicauda* ♂. In each photograph the chromosome pairs in synapsis show the actual chromomere differences as well as the similarities. A few lateral loop clusters were found on all the chromosomes. It should be pointed out that these preparations are not sections, but normally arranged chromosomes in their "chromogel," (see below) photographed at their isoelectric point of pH 4.6 after peeling off the nuclear membrane. Spatial arrangement in eggs are for the most part similar to those in somatic cells from tissue cultures, since the gel suspension mechanism can be shown to be identical, as illustrated in FIGURES 8, 9, and 10. But before considering the latter mechanism, let us return to the egg nucleus where colloidal relationships are easier to understand (*cf.* FIGURES 11 and 12).

It is often forgotten that in ovarian eggs of all vertebrates and of most in-



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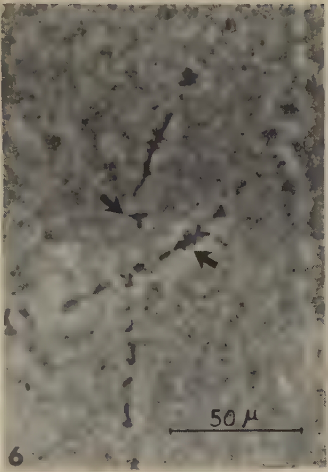

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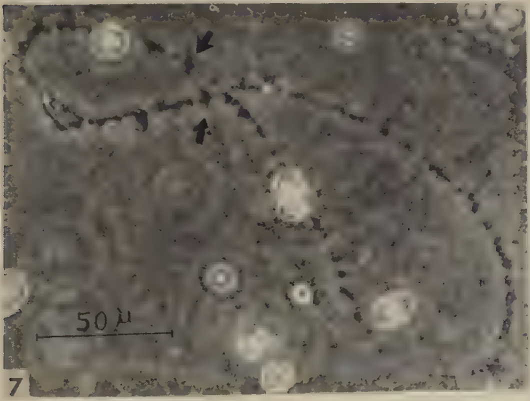

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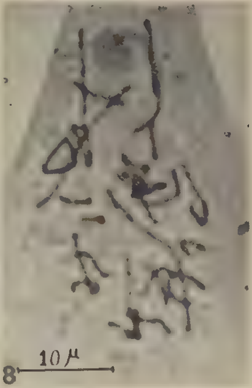

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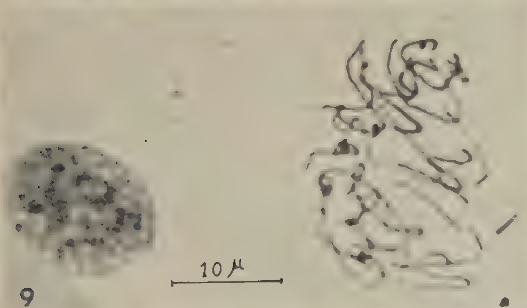

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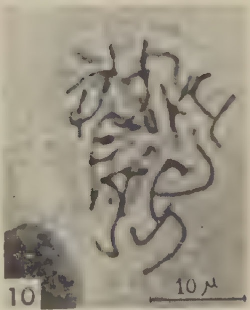

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FIGURE 5. Isolated living hybrid chromosomes from ovarian egg (*Triturus pyrrhogaster* ♀ × *Triturus ensicauda* ♂) showing pairs in synapsis. Animal furnished through courtesy of Doctor T. Kawamura.

FIGURE 6. Higher magnification of hybrid chromosome pair in synapsis showing details of chromomere differences as well as some similarities. Arrows point to apparently nonhomologous chromomeres.

FIGURE 7. Higher magnification of hybrid chromosome pair in synapsis showing details of chromomere similarities as well as some differences. Arrows point to apparently homologous chromomeres.

FIGURES 8, 9, and 10. Chromosome spreads of prophases prepared by Hsu Pomerat technique showing somatic synaptic condition. Culture of *Rana pipiens* kidney adenocarcinoma. Diploid number 26. Feulgen-light green.

vertebrates, a relatively enormous nucleus develops to provide a sufficient store of complex chemicals for the early course of the embryo, although the voluminous literature of the past 70 years attests this fact. As a more modern example, the germinal vesicle of the full-sized human egg was found in 17 cases to average 37μ in diameter and therefore approximately 26,000μ.[3] In comparison the average diameter of a follicle cell nucleus was 9μ with an average volume of about 380μ^3. Consequently, the human egg nucleus volume must increase about 69 times during its growth period. In the case of the frog egg nucleus illustrated in FIGURES 11 and 12 the growth factor is of the order of 64. In a paper previously presented before this Academy (1950) a mechanism for such volume changes was proposed, but the full implication is only now becoming apparent. The earlier explanation briefly was as follows: a primary oöcyte nucleus of normal somatic size starts growing *pari passu* with its cytoplasm. When the egg reaches one fourth to one third of its full diameter the chromosomes attain their maximum length (some over 750μ), because they are longitudinally stretched by being imbedded in a slowly hydrating gel. This structure was previously called the "chromosome frame. However, as this term is a clumsy one, we should now propose a simpler and specific one, *chromogel,* to describe the colloidal framework from which the chromosomes may never normally escape. The outer gel or peripheral layer, which in turn sustains the chromogel and keeps it from touching the nuclear membrane, may be termed the *nucleogel.* In egg cells the latter is highly labile and serves as a reservoir of chromosomal products and nuclear sap. In somatic cells the nucleogel is entirely absorbed into the chromogel and, since the interphase chromosomes are usually extended throughout the nucleus, therefore it is insignificant or totally lacking.

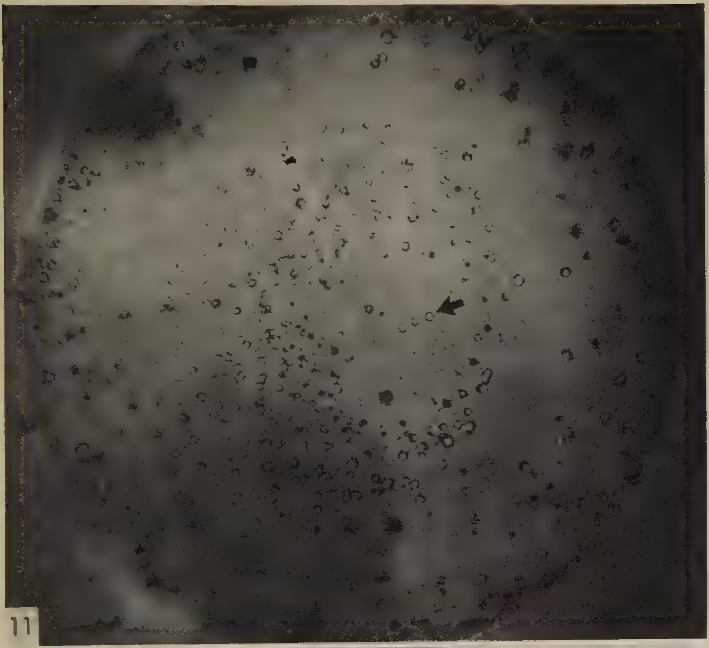
Colloidal properties of the chromogel are important for any theory of the nucleus. Although much information is still lacking, certain properties are easily recognizable in many phyla and in many different types of cells. Its most general characteristics are as follows:

(a) It may swell, shrink, or dissolve under physiological conditions.

(b) Its *outer layer* is particularly susceptible to transformation into fibrous, elastic and contractile elements. Under slow coagulation with $CaCl_2$ 0.001 M it can produce a monaster pattern in the mature frog germinal vesicle.

(c) It visibly transforms into spindle substance, and at least appears to arise by hydration from residual spindle cones after telophase.

We may conclude that the chromogel is the only apparatus by which chromosomes are moved about in the normal cell. It seems rather strange that this colloidal structure described clearly by the early cytologists and recognized in E. B. Wilson's classical textbook as the "karyosome" should have been so long ignored. Its significance for this conference is that it leads simply and directly to a new composite hypothesis of mitosis, which can be designated as the modified Hydration Theory. As will be shown below, it is quite different from the hydration and hydrodynamic theories summarized by Schrader (1953) and those mentioned by Hughes (1952). The diagram in FIGURE 13 illustrates our hy-



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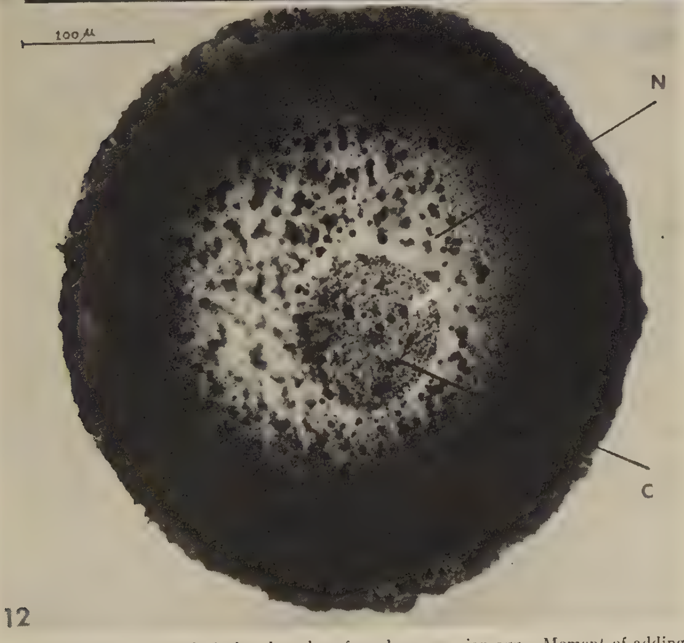

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FIGURE 11. *Rana pipiens.* Freshly isolated nucleus from large ovarian egg. Moment of adding solution of Janus Green 1:3000. Note central mass of nucleoli surrounding transparent chromogel.

FIGURE 12. Same nucleus at identical magnification 72 hours after addition of Janus Green. Note central spherical mass [= Chromogel (*C*)] containing chromosomes. Arrows indicate same three nucleoli in each photograph. Concentric unstained area indicates extent of nucleogel (N).

pothesis, which is based not only on experiments with amphibian and teleost eggs, but also on the important findings of J. G. Carlson on insect spindles. It is also in harmony with measurements made from the phase-contrast motion picture film of meiosis in the grasshopper spermatocyte produced by Kurt Michel (1942; United States Army Medical Training Film PMF 5036), and with some of the observations of Ris (1949).

It is of interest to outline, even with obvious imperfections, the colloidal stages from a morphological point of view by which the nucleus divides. Following the precedent in Schrader's revised monograph, consideration of cytoplasmic events is omitted. The account is intentionally generalized.

(1) Initiation of division may depend on a centriolar mechanism or may not. In tissue cultures of epithelial kidney cells and in ovarian theca cells, the focalized spindle suggests clearly a contractive center. However, this factor is completely lacking in first reduction divisions of the frog egg.

(2) Following prophase swelling, the chromogel contracts. Its more viscous outer coat carries the chromosomes to the region of the metaphase plate. A marked circumferential swelling has been noted from phase-contrast motion picture analysis, as mentioned above, in the equatorial region of the spindle. This factor must now be taken into account. It is difficult to conceive of any force that would produce this effect, except the obvious one resulting from polar compression. Nevertheless, we must admit there is little information on this critical and hitherto almost completely overlooked event.

(3) The beginning of chromosome separation apparently starts with molecular repulsions. But by the moment visible splitting starts, surface-controlled motion begins. In comparable material, Boss (1953) describes this movement as a gliding of the chromosomes along the spindle. Carlson refers the initiation of motion to the spindle surface. Our theory explains this initial motion as a loosening of the outer viscous shell of the chromogel accompanied by slow contraction of the polar cones. If kinetochores exude a specifically denaturing enzyme or agent, such a motion might well be expected in a stretched hemisphere or cone. Again it might result from a similar coagulant or denaturant emanating from a centrosphere, or from both simultaneously. Our theory attempts to explain the fact that no "traction fibers" as such are visible on the basis of earlier hydration experiments (Duryee 1941), and at the same time it accounts for motion toward the poles before spindle elongation. It likewise accounts for heavy visible fibers in the spindle material after mild dehydration or after the addition of standard fixatives. The theory allows for metabolic or enzymatic explanations, such as are brought forward by other authors in this volume, including Murray, Biesele, and Lettré. It avoids the difficulties of an "emerging thread" from the kineotchore. As shown in the diagram in FIGURE 13, it accounts for the prominent "notch" at the focalization point of the spindle, as well as for the tapering or focalization seen in many types.

(4) Late anaphase motion is clearly dependent on *oriented swelling* of central spindle micelles. No other force so universally fits the observations on plant and animal cells, accounting for such diverse phenomena as thrusting the ends

of the spindle against the cell wall, or continuing after heavy irradiations or microneedle stresses, or the gradual decrease in central or interzonal viscosity. It is not yet known what chemical type of micelle, or tactoid, could hydrate more in one dimension than in another. Nevertheless, this appears the most probable conclusion.

(5) At telephase the sharply defined contraction of chromosomes is almost pyknotic in some types of cells. That this development is a contracted chromogel phenomenon is strongly suggested by two experiments. Heavy irradiation (2000 r) of immature *Triturus* eggs will centrally clump the chromosomes into a telephase-size sphere (Duryee 1949). Treatment of cultured cells by hypotonic media, recommended by Hsu and Pomerat (1953), expands the telephase chromosomes radially. Critical examination of reconstituting nuclei, as illustrated in FIGURES 14 and 15, shows a progressive hydration of the chromogel in a thinly surrounding layer or nucleogel. Extremely strong support for this concept comes from the several cinéphotographic analyses presented at this conference, particularly from the clear film sequence made by Doctor J. Frederic of the University of Liége, in which telophasic reconstruction of the chromogel and radial separation of the daughter group of chromosomes from residual spindle substance are optically proved.

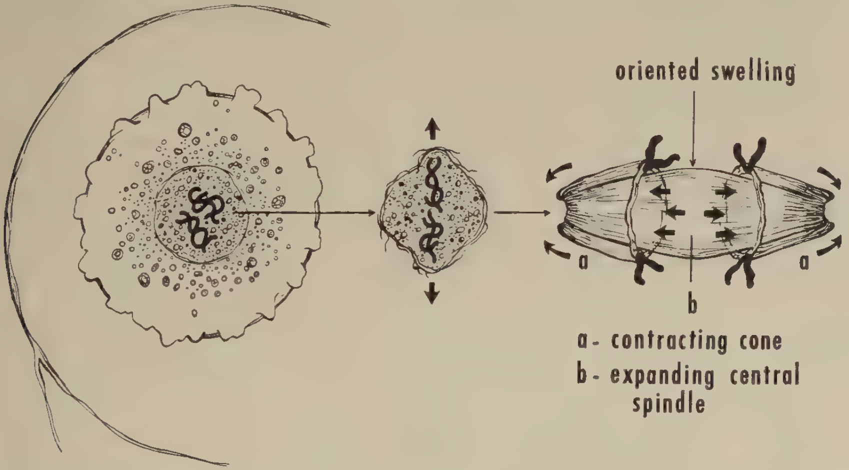
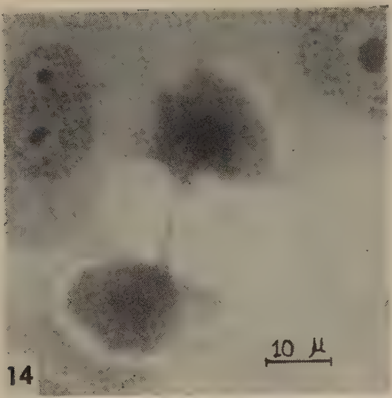


FIGURE 13. Diagram illustrating composite hypothesis of mitosis. At left is indicated the intranuclear contraction of chromogel, the viscous shell of which later carries chromosomes to region of metaphase plate. At center is shown circumferential swelling in equatorial region of the spindle. As shown at right, there is a splitting of the outer viscous shell of the chromogel, accompanied by slow contraction of the polar cones, plus "oriented swelling" of central spindle micelles.

In summary it can be said that the Modified Hydration Theory accounts for chromosome motions on the basis of a pushing or oriented swelling mechanism. This may or may not be aided by a pulling action of the outer chromogel remnant provided such a factor is present. For the chemical factors controlling these complex forces, the reader is referred to subsequent papers in this volume.



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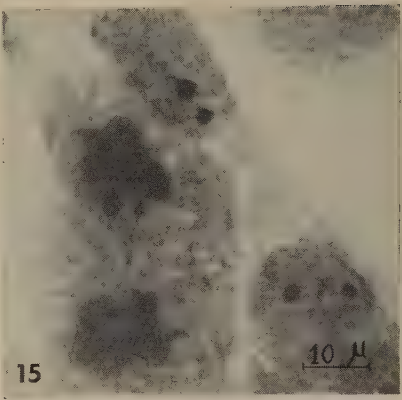

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FIGURE 14. *Rana pipiens*. Telophase of kidney adenocarcinoma cell in culture. Note clumped chromosomes in chromogel. Bouin, H & E.

FIGURE 15. Reconstituting nuclei following mitosis of malignant cell showing hydrating chromogel continous with spindle remnant. Paired nucleoli have now reappeared. Sublimate-acetic, H & E.

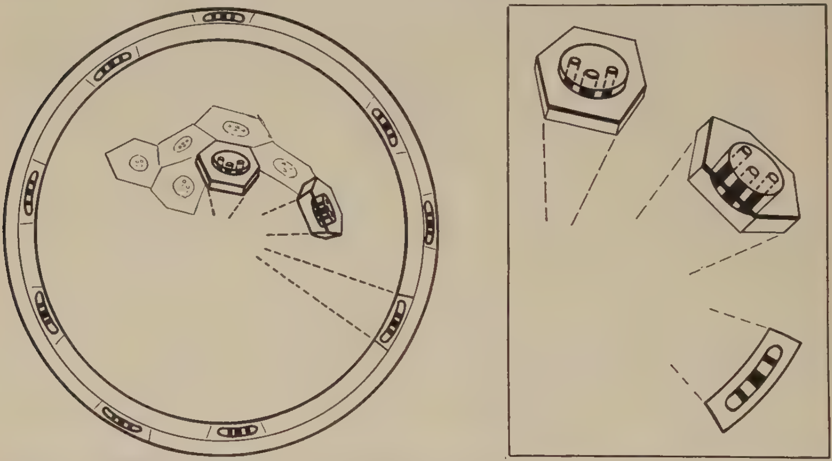

FIGURE 16. Diagram of theca cell nuclei surrounding an ovarian egg. Arrangement of rodlike chromosomes is perpendicular to the surface of the egg. Ends of rods are closely applied to inside of nuclear membrane. Chromosomes are held in position by colloidal matrix (chromogel).

Chromosomes in the Interphase Nucleus

In confirmation of the analysis presented above, two sources of evidence from interphase and prophase nuclei are available. The first is a special condition recognized only in theca cells surrounding the transparent immature eggs in an ovary. The second arises from application of the Hsu-Pomerat technique to kidney epithelial cells in culture.

Theca Cell Nuclei. As indicated in the diagram in FIGURE 16, flat, platelike cells form a two-layer theca around the oöcyte. Normally such nuclei are completely transparent and devoid of optical structure. Addition of hypertonic Ringer's solution, or β-radiation from P^{32} injected five days previously,* or decrease in pH of the medium to 5.0, all gave a similar result—namely, visualization of rodlike elements in the nuclei. It was found that these threads were uniformly oriented perpendicular to the surface of the egg beneath. In many thousands of theca cells examined, the structure was found surprisingly uniform. Feulgen staining of the rods was positive, corresponding to mitotic chromosomes occasionally found. It was concluded that these structures were interphase chromosomes held uniformly in a type of chromogel where the ends of the rods were closely applied to the inside of the nuclear membrane. Of further interest was the fact that the diploid number 22 was frequently counted, although the stastitical variations centered about 19 ± 5. The reason for this unusual orientation of chromosomes in somatic nuclei is unknown. It could be related to some overall permeability problem of the theca since more than 80 per cent of the surface area surrounding the egg appears blanketed by flat nuclei.

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* Injections into each *Triturus pyrrhogaster* consisted of 0.5 cc. Ringer solution made up with P^{32} to give an estimated activity of 0.04 mc./cc.

Somatic Synapsis in Cultured Cells. In many instances where Hsu-Pomerat (1953 hypotonic medium was applied to cultures of both frog kidney adenocarcinoma and of normal cells for 20 minutes prior to fixation, chromosome "spreads" were observed. These spreads, chiefly from prophases, were treated routinely with Feulgen or hematoxylin stains. In 14 particularly clear preparations, the chromosomes were found in synaptic pairs, parallel or twisted about one another, or in characteristic ring formations. Chromosome counts averaged 26 ± 1, the diploid number. Examples of Feulgen stained spreads are illustrated in FIGURES 8, 9, and 10. In a previous communication (1950) the number and individual pair characteristics of chromosomes in isolated frog egg nuclei were given as: 4 ring-shaped, 4 medium, 3 long, and 2 short pairs, designated by the letters Q, R, S, and T, respectively. For comparison the chromosomes in FIGURE 8 may be analyzed into: 4 rings (2 broken), 4 medium pairs with at least one chiasma, 3 long pairs (1 separated), and 2 short pairs. The implications of this condition are far-reaching. Chromosomes in these neoplasia are very many cell generations removed from the egg. Consequently, close approximation of the synaptic, *i. e.*, associated, homologues cannot possibly have come about by chance. Chromosomes twist about one another and the rings open out specifically because the controlled hydration of the chromogel has changed to a viscous solution. The conclusion is inescapable that chromosome pairs remain in association throughout life, and that this association, in turn, must depend on nuclear mechanics.* A stable chromogel system with flexible attachments between homologous kinetochores seems the most logical solution.

The Nuclear Membrane

One of the most remarkable cell organoids is the nuclear membrane, yet it is not listed as such in any text book. In the eggs of vertebrates the structural features are most developed. Surface sacs or pouches in the frog for example, as shown in FIGURE 17, persist in the growing oöcyte for over seven months. Even in the amoeba, as proved by the electron micrographs of Bairati and Lehmann (1952), the pattern of pores is a fixed one. Contrary to Callan's (1950) view, the nuclear membranes of both urodele and anuran eggs behave as a single-layered, physiological unit. Through phase-contrast microscopy, the living membrane was found to be composed of a mosaic of lenticular vesicles ranging in diameter from 5 to 50μ and projecting 5 to 30μ above the main surface. These vesicles are clearly seen in FIGURES 18, 19, and 20. The larger, internally patent sacs (50 to 100μ) characteristic of American and European frogs and toads are missing in urodele amphibian nuclear membranes, and are also lacking in nuclei of fish and mammalian ova. It is of interest that the smaller vesicles are always present, since they constitute the best example of a true mosaic living membrane.

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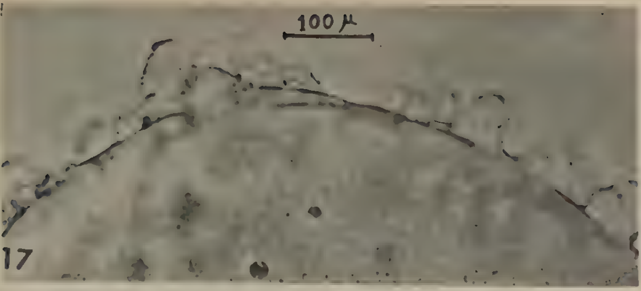
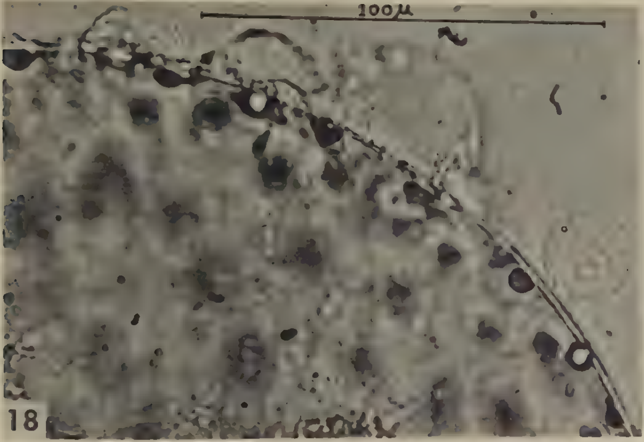
* Doctor Hsu kindly examined our microphotographs and agrees with our interpretation that the distribution of chromosomes is not completely random. It is noteworthy that some of his own published photographs mammalian material also show this association of chromosomes in "pairs."

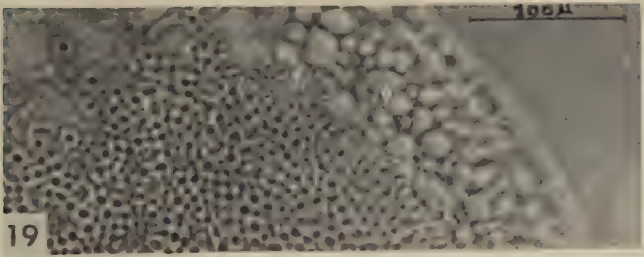
In this introduction to the subject, it is possible to mention briefly only some of the experimental attacks on problems of the cytoplasmic-nuclear interface. By use of quantitatively controlled volumes of 0.001 N. NaOH it was possible to dissect membranes of isolated nuclei chemically. FIGURES 19 and 20 illustrate successive stages in the disintegration process. In FIGURE 19 the vesicles more protected from the OH^- ion had barely begun to swell, while those on the curving surface were coalescing rapidly. FIGURE 20 shows greater detail in changes on the lipo-protein surface. Its lipo-protein nature was shown by solubility in trypsin, solubility being incomplete in pepsin and ether solutions. If not reversed promptly with appropriate concentrations of H^+ ions, a significant shredding phenomenon took place. This shredding can be seen in FIGURES 21, 22, and 23. The photographs were taken at approximately 60-second intervals. It will be noted that coalescence of vesicles is followed by a relaxation of the protein fibers, which spin out into threads. Even at the stage shown in FIGURE 21, addition of dilute acid drew the fibrils back into a smooth sheet with minimum area at the isoelectric point of pH 4.5. Other reagents such as $Ca(NO_3)_2$ and $CaCl_2$ in concentrations from 0.001 to 0.8 M gave, graded reactions to the sheet, shredding occurring in the highest concentrations, but not in the physiological range.

Interesting differences in the reactions of live and dead membranes were noted with reagents such as 0.1 M H_3BO_3, $Pb(NO_3)_2$, and $NaVO_3$. It was concluded that a layer of residual cytoplasm maintained the vital characteristics of *isolated* membranes, allowing anion, or swelling, effects to predominate over cations, effects which are recognizable flocculations. Washed or inactivated membranes showed reverse results. Nucleogel swelling may be conveniently designated *alpha* and nucleogel flocculations designated *beta* type reactions, respectively. When these reagents were micro injected into living cells against the nuclear membrane, *alpha* type reactions followed. It was concluded that membrane integrity must be maintained in the living cell to allow excess negative colloidal charges to accumulate in the nucleogel.

Whenever the nucleus is damaged so that positive ions in great excess can enter, the nuclear colloids are flocculated and death processes ensue. On the other hand, in a normal prophase nucleus, not subject to harsh reagents, the colloidal electrical charge unbalance is much more delicate and could be ex-







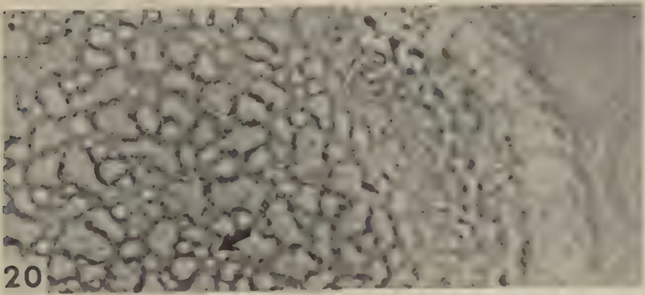
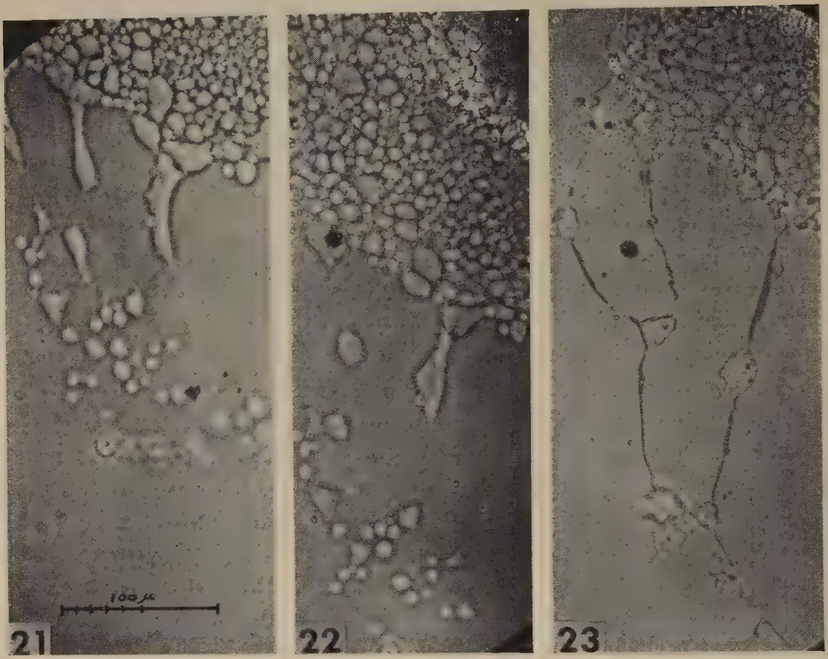


FIGURE 17. *Rana pipiens.* Detail of organoid structure in nuclear membrane showing pouches. Note flocculating nucleogel and two nucleoli. Nucleus isolated from full-sized ovarian egg.

FIGURE 18. *Triturus pyrrhogaster.* Detail of nuclear membrane showing four different stages in extrusion of nucleolar contents. Note end of chromosome thread in lower left corner and nucleoli adherent to inside of membrane. Nucleus isolated from immature ovarian egg.

FIGURE 19. *Triturus pyrrhogaster.* Nuclear membrane from isolated immature ovarian egg showing beginning effects on vesicle disintegration 90 seconds after the addition of 0.001 M NaOH.

FIGURE 20. Later stages of vesicle disintegration by 0.001 M NaOH. Arrow indicates vesicles of original size which have not yet begun to coalesce.



FIGURES 21, 22, and 23. Later successive stages of chemical dissection of the nuclear membrane with 0.001 M NaOH at approximately 1 minute intervals. Formation of protein fibers following coalescence of vesicles as shown in FIGURES 19 and 20.

pected to play a vital role in balancing hydration changes in both nucleogel and chromogel during mitosis. It should be borne in mind that colloidal charge alone is not the prime cause in mitosis, as these experiments showed, but it is an important factor that can account for the power of the outer rim of the chromogel to draw the chromosomes together.

Nuclear Secretion

No theory of the nucleus would be complete without attempting to explain its primary role in translating genetic information into cytoplasmic behavior. Perhaps this is the most difficult question which faces the student of the cell. In our vast ignorance no one set of conjectures and analyses will suffice. It may be useful, however, to point out some approaches to the dominant problem.

In the first place, there is concrete evidence accumulated by numerous geneticists, cytologists, and biochemists that DNA is localized on chromonemata and is the chief chemical component of enzymes and some viruses. In the egg cell it has long been known that there the chromosomes reach maximum size and development. Specifically it has been shown by one of us (Duryee 1941) that egg chromosomes synthesize nucleic acid branches in the form of lateral loops from chromomere loci, and that these loops slough off, accumulate in the nucleogel, and become distributed into egg cytoplasm at the time of germinal vesicle breakdown.

Another type of nuclear contribution is via nucleolar extrusion through the nuclear membrane. As far back as 1908, Walker and Tozer clearly observed and correctly interpreted their findings in planarian cells and in rabbit leucocytes. An extensive literature supports their basic conclusion. It has been frequently observed in our own work with amphibian oöcytes that some nucleoli containing DNA reach the nuclear membrane and evert their contents into the cytoplasm. For an unusually clear illustration of this process, attention is called to FIGURE 18, where four stages of nucleolar contents can be seen traversing the membrane. The number and characteristics of similar egg nucleoli are shown in FIGURES 11 and 12.

A third and altogether different type of nuclear contribution has been noted by a few tissue culture investigators. This method includes minor fragmentations and blebs of extruded nuclear material as illustrated by Pomerat's cineanalysis in this conference. We have become aware of a variant of this method, which deserves illustration and comment. As Lucké (1951) has so well shown, the frog kidney adenocarcinoma cell both *in vivo* and *in vitro* characteristically produces large eosinophilic inclusion bodies. As seen in our cultures, these bodies were traceable back to the nucleus. In nearly every field, one or more examples of inclusion body material emerging from the nucleus could be found. The principle is illustrated diagramatically in FIGURE 24. With microneedles we have determined that these viscous, semisolid bodies can stem directly from the nuclear interior. This type of inclusion body has also been repeatedly observed in rapidly dividing normal cells, as in the lung, bladder, and kidney. These bodies are rare, however, in quiescent cells, and never as numerous or as large as in malignant tissue. The striking size and frequency of occurrence is so great in the latter that the conclusion is warranted that nuclear extrusion is a major method of nuclear function. Further examples are illustrated in FIGURES 25, 26, 27, and 28. In multinucleate giant cells, where up to 55 nuclei have been observed, inclusion body material often accumulates in a central nuclear area. FIGURE 28 clearly demonstrates this condition. Where binucleate cells occur, almost simultaneous extrusions can occasionally be demonstrated. An example is FIGURE 27. It is important that this process can be observed also in the living cell, although nuclear outbursts are surprisingly rapid and seldom take more than a few minutes. Continuous extrusion tangential to the membrane surface could account for the phenomenon of nuclear rotation, as observed by Pomerat and by Tahmisian. FIGURE 25 shows a living tumor cell where the inclusion body was seen to leave the place of formation and migrate 30μ into the cytoplasm. Of great significance is the fact that inclusion body material can often be identified *within* the nucleus and frequently in conjunction with the nucleolus. There is as yet no final proof of any causal relationship, but we have many preparations showing outpouring of the nucleolar interior.

The cytochemistry of similar inclusions in Shope fibroma cells of the rabbit has recently been investigated by Fisher (1953). A superficial resemblance between the rabbit and frog material exists. Fisher found no DNA or RNA present, and no glycogen, but protein tests were all positive, especially the ninhydrin-Schiff reaction. He concluded that the material was chiefly glycoprotein and cytoplasmic in origin. There is perhaps little likelihood that the inclusions of these differing virus tumors are the same. But there is still the possibility that both types of nuclei do produce protein, and that nuclear extrusion through the membrane is of more general significance. In amphibian material there is no doubt that nuclei can extrude considerable volumes of material, and this material might contain RNA. Even in the more limited amounts in normal quiescent cells, this extrusion could be of critical importance. Further experiments and a wide search for similar events in other laboratories would seem worth while. If DNA does control RNA production, and this production in turn furnishes the template for protein, and if the nucleus becomes known as the principle secretory organ of the cell, a long step will have been made toward understanding cytoplasmic behavior.

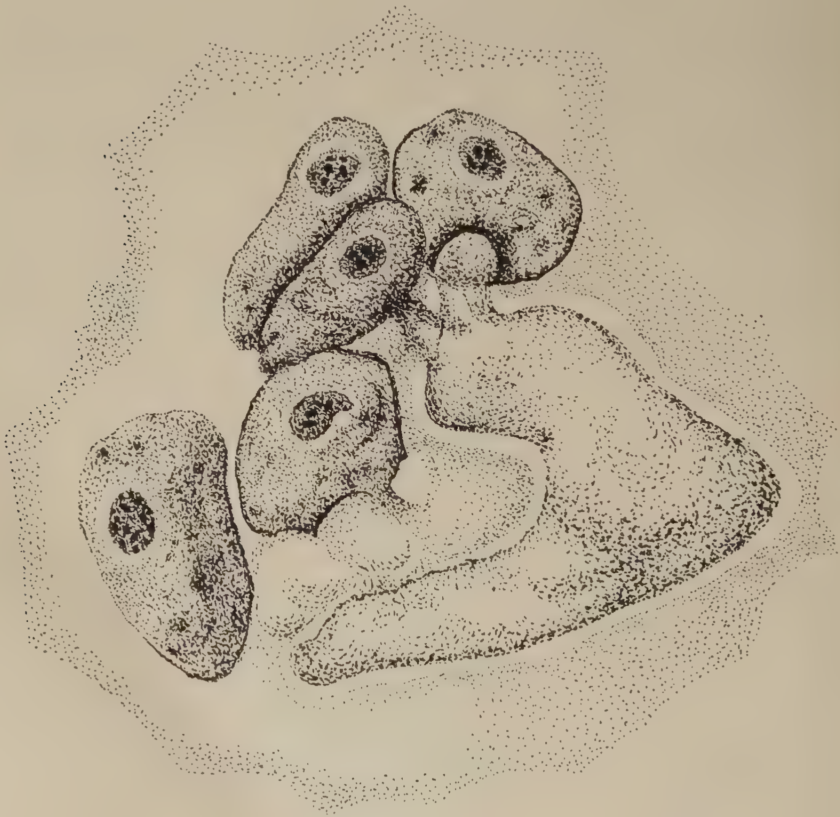
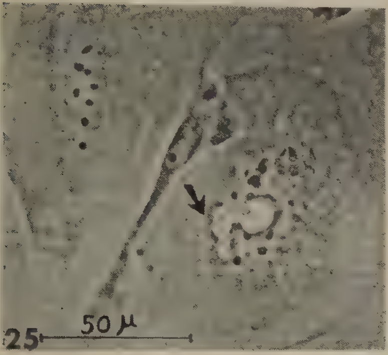
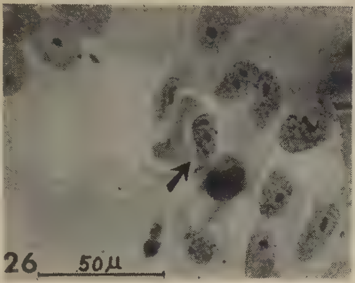
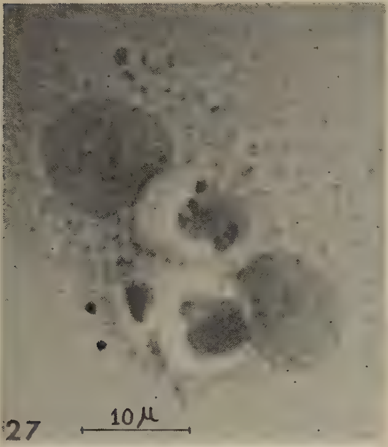


FIGURE 24. Semidiagrammatic representation of inclusion body emerging from nuclei of a cell in tissue culture. Drawn from a photograph of a frog kidney adenocarcinoma cell stained with H & E.







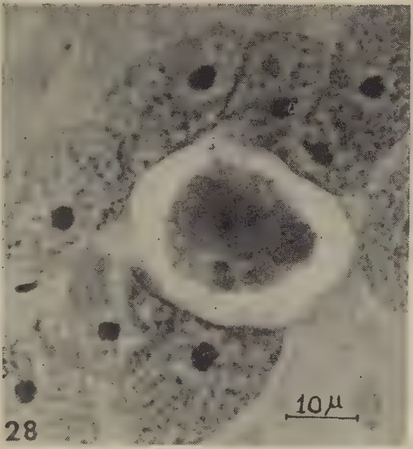


FIGURE 25. Living *Rana pipiens* kidney adenocarcinoma cell in culture. Inclusion body (→) extruded from nucleus now moving out of nuclear area.

FIGURE 26. *Rana pipiens* kidney adenocarcinoma cell in culture with inclusion body emerging from nucleus. Bouin, H & E.

FIGURE 27. *Rana pipiens* kidney adenocarcinoma cultured binucleate cell showing inclusion bodies derived from each nucleus. Sublimate-acetic. Feulgen-light green.

FIGURE 28. *Rana pipiens* kidney adenocarcinoma multinucleated giant cell showing central inclusion body shortly after extrusion from the nuclei. Culture fixed in Bouin, H & E.

Discussion

The physiological advantages of nonuniform organlike structures in a single protoplasmic system should be considered not only in their relationship to one another, but to the whole organism. Thus chromosome threads, which are organoids in themselves, stretch out in the interphase nucleus because of the hydrating chromogel. This extended hydrated condition undoubtedly offers additional and more effective surface area. It should be recalled that Dodson (1949) calculated the specific surface of *Amphiuma* oöcyte chromosomes as 108.37 square meters per gram of tissue. The fact that countable threads, agreeing closely with diploid values, can be seen in living ovarian theca cells and in some tissue cultures of kidney epithelium should go far to resolve the old problem of what becomes of interphase chromosomes in a transparent nucleus. Whether the mass preparations obtained by grinding techniques represent true chromosomes is debatable. It appears preferable to isolate them singly with micromanipulation. Another geometric configuration of functional importance is the relationship of nucleoli to the surface membrane. In oöcyte nuclei their migration outward from their site of origin on the chromosomes depends on the expanding chromogel. These transport structures can evert their contents through the membrane because of its semiliquid character and their own coacervate properties. Nucleoli of kidney tumor cells in culture can also become adherent to the inner surface of the nuclear membrane. It is scarcely necessary to discuss comparable situations in the cytoplasm, where mitochondria and centrifugally separable components are better understood.

With regard to general applicability of information from germ cells to somatic ones, it should be remembered that the former are prototypes for all others. A germ cell nucleus with its magnified features can be useful in understanding somatic nuclear behavior. For example, study of nucleogel and chromogel changes following irradiation of frog eggs forms a rationale for understanding the mechanisms of pyknosis, karyolysis, and related pathological changes in somatic cells (Duryee 1949). Another illustration is in the report by K. Dan (1952) that the egg of the echinoderm crinoid, *Comanthus japonica*, contains a spindle-forming mechanism similar to that described in our earlier paper for amphibia.

Mention should be made of recent theories of the nuclear membrane. It is of interest that pore systems similar to those described by Callan and Tomlin (1950) in nuclear membranes of toad eggs have been reported by Bairati and Lehmann (1952) in *Amoeba proteus*. The latter investigators postulated a regulation of nuclear permeability by the lipid rich continuum and considered the porous layer to be mechanical only. Our experiments on washed nuclei show that the permeability changes arise not from removal of a lipid layer, but from denaturation of the protein. Some extrusions appear to be able to escape because of the layer's semiliquid properties. The conclusions of Callan and Tomlin on coagulated membranes are not supported by our results.

Attention should be focused on the growing problem of nuclear metabolism. Our finding of large outpourings from the nucleus makes inescapable the conclusion that corresponding amounts of precursor material must have entered earlier. The theca cell condition described above argues for a *flow* of metabolites *through* the nucleus. In human ovarian eggs, it was estimated that 3000 theca cell nuclei surround the ovum in the corona radiata and are in direct communication with egg cytoplasm via dissectable tubules through the zona pellucida. This previously unpublished observation is in agreement with the hypothesis that the nucleus is the chief source of protein in the cell. In 1949 Abelson and Duryee showed by radioautographs that nuclei of frog eggs were capable of concentration Na^{24} by a factor of 2 over the cytoplasm in 30 minutes. There is evidence that these nuclei concentrate P^{32} by an even greater factor. It seems reasonable to expect that the search for nuclear contributions to cytoplasmic proteins will go forward in many laboratories on a larger scale, and that, conversely, interpretations of metabolic transformations in the cell will increasingly be made in terms of organoid structure.

Summary

(1) Organoid features of nucleus and cytoplasm have been considered from the general viewpoint of nonuniform areas in a complex protoplasmic colloid.

(2) The nucleus of the amphibian egg was shown to consist of two concentric colloidal spheres: the chromogel and nucleogel. The former, defined as the suspending mechanism for chromosomes, becomes incorporated into the spindle. Nucleogel, when present in somatic nuclei, becomes converted into cytoplasm at the onset of mitosis.

(3) At telephase the half-spindle residuum reconstitutes by swelling into chromogel and nucleogel.

(4) A new hypothesis designated as a Modified Hydration Theory for spindle action was presented. It is based on oriented swelling of the central micelles and contractions of the outer polar cones.

(5) Examples of somatic synapsis were shown to occur in both normal and malignant cells. These conjunctions were interpreted as evidence for persistence of chromogel-chromosome connections during the life history of the individual.

(6) The nuclear membrane of amphibian oöcytes was shown to consist of a lipo-protein layer covered with lenticular vesicles arising from nucleoli. This was related to the behavior of nuclear membranes of amphibian cells in tissue culture.

(7) Evidence for the major role of the nucleus as a secretory organoid in both normal and malignant cells was presented. Nuclear secretion was found more evident in the latter and correlated with cell multiplication and growth.

(8) Cilia differentiated in cultures of normal amphibian lung, of kidney and bladder tissue, and of kidney adenocarcinoma. Both synchronization and rate of beat were found dependent on interconnections of the basal granules. Ciliary motion could be briefly stopped by exposure to freezing temperature.

(9) A modification of the Earle perforated-cellophane technique is described for culture of amphibian cells.

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Discussion of the Paper

DOCTOR F. WASSERMAN (*Argonne National Laboratory, Lemont, Ill.*): I should like to commend Doctor Duryee for stressing the significance of physical changes in viscosity, hydration, *etc.* during the mitotic cycle. These aspects of mitosis have been somewhat neglected in recent years. An attempt should be made to correlate them with chemical processes, and not to forget them. Doctor Duryee's hypothesis concerning the anaphasic movement of the chromosomes is very similar to my own interpretation, recently discussed by Schrader in his monograph on mitosis under the title of "Viscosity and Hydration." An important question to be asked, in criticizing a hypothesis such as Doctor Duryee's, or my own, is whether it takes into consideration the variations of the mitotic process, especially those concerning the spindle. I believe the distinction of two types of spindles is justified: one that is formed and grows between the centrosomes and is preserved throughout mitosis, and the other type, which develops after the dissolving of the nuclear membrane. Corresponding to these two types we find the chromosomes either attached to the surface of the primary spindle or taken in between the two half-spindles, thereby occupying the entire equatorial plane of the spindle. Doctor Duryee's diagram seems to represent only this type of the primary spindle.

DOCTOR DURYEE: The splendid contributions of Doctor Wasserman to the theory of mitosis are well known. It might be pointed out, however, that the swelling action of micelles, together with contractions of the outer sheath can be applied to either type of spindle Doctor Wasserman mentions. There is no conflict of the theory as here presented with a centrosomal initiating mechanism found in certain cells. I find it preferable to regard all kinds of spindles as variants of a single type. Some kinds have a centrosomal trigger or actuating device, but the vast majority do not.

EFFECTS OF PENICILLIN AND STREPTOMYCIN ON STAPHYLOCOCCI IN CULTURES OF MONONUCLEAR PHAGOCYTES*

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Introduction and Analysis of Variables

The failure of antibiotic therapy to eliminate some infectious processes has led a number of investigators to study antibiotic and chemotherapeutic action on intracellular bacteria. In such studies the selection of the source of cells, the types of cells, the characteristics of the bacterial strain, the use of an autogenous, homologous, or heterologous system are important in the interpretation of the results. There are several host-parasite relations *in vitro* which should be considered:

The Cells

(1) Whether attached to solid surfaces or floating freely.
    (a) Differences in mechanisms of phagocytosis.
    (b) Differences in sampling.
(2) Whether multiplication is occurring.
    (a) Multiplication of certain cell types may decrease the parasite-cell ratio, whereas the absolute number of parasites may remain unchanged.
(3) Death of cells, or loss of cells by cannibalism.
    (a) May increase the parasite-cell ratio, whereas the absolute number of parasites may remain unchanged.

The Bacteria

(4) Whether multiplication takes place within the host cell.
(5) Whether bacteria die, yet retain morphology.
(6) Whether bacteria die and disappear.
(7) Whether multiplication occurs in the extracellular fluid.
(8) Continued phagocytosis.
(9) Egestion of bacteria.[1]
(10) Development of resistance to the antibiotic.
(11) Change of activity of antibiotic.

Review of Previous Work

Studies of *Brucella*, *Mycobacterium tuberculosis*, and staphylococci have been reported; a brief description of the culture techniques and some of their possibilities for information are described briefly below, but the aims of the investigators and the significance of their observations are reviewed elsewhere and will not be discussed here.

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* This study was supported by a contract with the office of the Surgeon General, Department of the U. S. Army.

Shaffer, Kucera, and Spink[2] described studies of *Brucella* within albino rat mononuclear cells gently shaken in a tube for a period of 24 hours. In this method a volume of the cell suspension is removed at 24 hours and cultured in agar for viable organisms. Another sample is treated with neutral red vital stain for determination of the viability of the cells, and a sample is smeared on glass for study of stained preparation. From this work there is produced a good study of the relative activity of streptomycin alone, streptomycin combined with sulfamerazine, streptomycin combined with terramycin and streptomycin combined with chloramphenicol. The number of viable *Brucella* counted at 24 hours showed that with streptomycin alone 11 per cent remained; streptomycin plus sulfamerazine, 6.4 per cent; streptomycin plus terramycin, 2.6 per cent; streptomycin plus chloramphemicol, 1.4 per cent. The remaining viable *Brucella* counted in heat-killed cells exposed to the same mixtures was 40 to 280 times lower and demonstrated the relative protection of *Brucella* from antibiotics by the living cell. This report showed no data on the change in the number of visible organisms within the cells, on the change in total cell count, or on the number of viable *Brucella* in those cells showing surviving *Brucella.*

G. B. Mackaness[3] and Mackaness and Smith[4] have studied the survival of *Mycobacterium tuberculosis* in rabbit mononuclear cells. These cultures are maintained between two coverslips 1.5 mm. apart held in a plastic support that allows diffusion of media from a surrounding reservoir. Here it is possible to observe the viability of the cells by direct microscopy and to remove the coverslip for a stained preparation to observe the numbers and conditions of bacilli. The mononuclear cells are not observed to multiply. After 2 to 3 days the organisms are seen to change morphology and multiply within the cells. After this time lysis of the mononuclear cells is rapid, and the cultures deteriorate. The survival of infected cultures is shown to be influenced by streptomycin and isoniazid. The percentage of macrophages containing viable organisms is assessed by transferring a sample of the cells from the chamber to solid media in slide culture and measuring the relative number of microcolonies to macrophage remains. Ten μg. of streptomycin in the medium controls the multiplication of organisms within the mononuclear phagocytes but studies on agar show that the relative number of mononuclear cells containing viable organisms is not substantially reduced. Isoniazid 1 μg./ml. produces a steady reduction in the relative number of cells with viable organisms. The addition of streptomycin 10 μg./ml. is shown to enhance this effect.

The technic of Suter[5, 6] allows infected cells to settle on glass coverslips. The coverslips are then rinsed and covered with formvar film. The coverslips are then placed on edge on the inside of a screw-capped vial containing a small amount of nutrient media. The side of the coverslip which is covered with cells is placed next to the wall and is bathed by a film of fluid rising between the coverglass and the wall from the media in the bottom. This technic does not indicate the numbers of viable organisms; counts from the stained coverslips, however, show the numbers of organisms per infected cell. The tubercle bacilli apparently multiply in guinea pig monocytes in the presence of concentrations of streptomycin which are inhibitory for cell-free organisms if the assumption is made that there is no significant change in the total number of mononuclear phagocytes during the period. This technic has been further used to show the property of cells from BCG-vaccinated animals to prevent this intracellular multiplication.

Survival of Staphylococci in Tissue Cultures Treated with Antibiotics

At the meeting of the Tissue Culture Association in March 1952, the author reported that staphylococci are protected to some extent from penicillin and streptomycin by mononuclear phagocytic cells.[7] Recently investigation of some quantitative aspects of the elimination of these staphylococci in cell cultures treated with penicillin over a six-day period have been carried out and are reported below. During these studies it became evident that an understanding of the cellular activity in the elimination of staphylococci was essential in the part played by an antibiotic. Consequently, short-term experiments designed to obtain information concerning effects of cellular activity on staphylococci were undertaken. For clarity, the latter studies are reported first.

Survival of staphylococci in mononuclear phagocytes without antibiotic. The tremendous potential of staphylococci for extracellular proliferation is one of the most troublesome factors in the assessment of their intracellular survival. This proliferation provides for continuous ingestion of staphylococci and comprises a conclusion on the survival time of staphylococci found at any time of sampling following the initial phagocytosis period. In an attempt to mitigate this confusing factor, to allow the macrophages to function on a surface and to provide a random sample of the initial inoculum, the following method has been tried. This method provides a continuous flow of fresh media over the cells to reduce the accumulation of staphylococci in the extracellular fluid.

Special apparatus. For each sample two tubes were prepared from 7-mm. Pyrex glass, one short tube containing the infected cells and one longer tube as a reservoir from which nutrient media can continuously supply the first (FIGURE 1). The tubes for containing the cells are prepared from an 11-cm. length of tubing ground to flatness on both ends and cleaned. This tube is heated in the center in a fine oxygen flame and pulled to form two 5-cm. tubes with a tip at one end at an angle of approximately 45°. This tip is sealed. Five mm. above the tip the tube is deeply scored for subsequent breaking. The ground end is fitted with a piece of tygon tubing to serve as connector for the feeder piece. The feeder reservoir consists of 14 cm. of the same tubing, ground to flatness on one end. Approximately 1.5 cm. above the ground end, this tube is heated in the flame and pulled to form a capillary that permits about 3 mm.

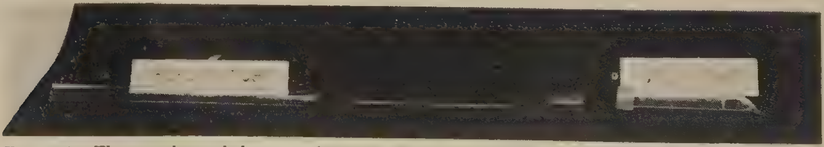

FIGURE 1. The two pieces of glassware that are used in maintaining the cells. The feeder tube on the left is the reservoir for nutrient media. The tube on the right is for maintenance of cells adherent to its inner wall. The section of tygon tubing for connection to the feeder and the score mark for subsequent breaking are shown.

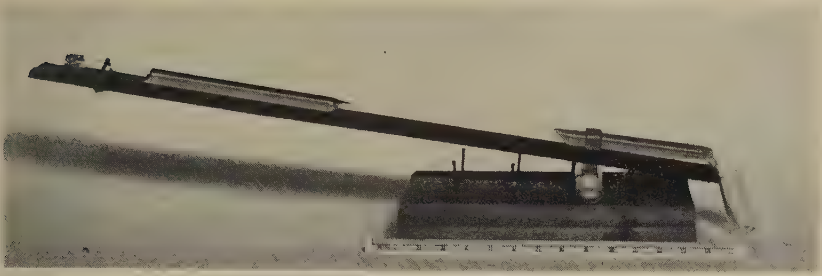

FIGURE 2. Apparatus assembled for flow.

per minute decline in water level when held vertically. This procedure allows for adjustment on an inclined rack for flow of media at approximately one ml. per hour (FIGURE 2).

At the time of assay, a rubber squeegee is used to remove the cells from the inner wall of the glass tubing. This device is prepared by fitting a section of latex rubber tubing to the slightly enlarged end of a 3-mm. glass rod. Backing the rubber ring is a short section of a 5-mm. glass tubing cemented to the glass rod.

Source of Staphylococci and Mononuclear Phagocytes

All studies of staphylococci have been conducted with a strain of *Staphylococcus aureus*, isolated from a fatal case of pyelonephritis, which produces a firm coagulum in human plasma and a weak coagulum in rat plasma. It produces a large zone of hemolysis on rabbit blood agar and a narrow, well-defined zone of hemolysis on human and horse blood agar. Its *in vitro* sensitivity to streptomycin is 0.5 μg./ml. and to penicillin 0.04 μg./ml. Prior to the experimentation, 0.1 ml. aliquots of a 5-hour nutrient broth culture of this organism are quick frozen in sealed ampules. These ampules are kept in a dry-ice storage cabinet, and one ampule is opened for use at the start of each experiment.

The 80 to 90 per cent mononuclear phagocytes are obtained in a suspension of cells from the pleural space of 250- to 300-gram albino rats which are prepared as follows: three injections of 0.3 ml. into the pleural cavity are made at seven day intervals. The material injected is a suspension of staphylococci prepared as described in reference No. 8 and killed by formalin at a final concentration of 0.25 per cent. The cells are removed seven to ten days after the last injection. For controls in these experiments, animals are similarly prepared with a nonspecific vaccine from a diphtheroid organism.

Procedure. An aseptic technique is used throughout. The animals are bled from the inferior vena cava. Regularly 8 to 10 ml. of blood can be obtained for deriving the serum for the experiment. The abdominal aorta is cut to exsanguinate the animal. The right thorax is shaved and treated with iodine solution. After the cessation of respiration four ml. of Hank's Balanced Salt Solution (BSS) containing approximately 15,000 staphylococci per ml. is injected into the pleural space. The animal is shaken at intervals, and after 10 minutes nearly 2.5 ml. of the fluid is removed with a Pasteur pipette through a thoracotomy hole. This fluid is combined with fresh normal rat serum to make 40 per cent serum. Three of the 5-cm. glass tubes previously described are filled with this mixture of cells and staphylococci. The tubes are stoppered and placed in the incubator for phagocytosis to proceed, and for the cells to become adherent to the glass wall.

During the interval the feeding tubes are filled with BSS without bicarbonate and stoppered at the upper end to prevent backflow of any staphylococci during the connecting process. After the primary incubation, two of the cell tubes are set up for flow in the following manner. The sealed tip of the tube is broken, the stopper is removed, and the fluid is allowed to run out of the tip. The tube is immediately filled with neutralized BSS and the ground end is connected to the ground end of the feeder tube. The apparatus is placed on an adjustable inclined rack, the stopper replaced by aluminum foil, and the flow is regulated by the incline. The nutrient fluid, 40 per cent autogenous serum in BSS, is supplied to the feeder tube. The apparatus is incubated at 37° C. This time is designated as "zero" hour.

The remaining tube containing infected cells is immediately sacrificed as follows: the contents of this tube are allowed to drain. The lower lip is broken off, and the rubber squeegee is inserted in the upper end. One ml. of BSS is used to fill the tube, and any remainder is placed in a short collecting tube 12 mm. in diameter and 25 mm. long. Materials for assay are shown in FIGURE 3. The squeegee pushes the BSS before it, and also a large sample of the cells from the wall of the cell tube, into the collecting tube. These cells are dispersed by drawing 20 times into a pipette. The suspension is distributed as approximately 0.4 ml. to a shell vial having a round coverslip covering the bottom; exactly 0.1 ml. to a previously incubated blood agar plate and promptly spread over the surface; approximately 0.25 ml. to an iced lusteroid tube for exposure to sonic vibration (Raytheon 10 kc. oscillator) to disrupt the cells, from which exactly 0.1 ml. is similarly plated on blood agar. The remainder is used for smear on glass slide. The hemacytometer pipette takes its sample from the shell vial The shell vial is promptly centrifuged at 1000 r.p.m. for 10 minutes. The coverslip is removed, fixed in two parts Zenker fluid and one part methyl alcohol for five minutes, rinsed in water, and stained for staphylococci and cell details.*

The flowing specimens are similarly sacrificed and assayed at two and one-half hours and five and one-half hours.

Observations and Discussion

Data concerning the survival of the bacteria among the mononuclear phagocytes can be obtained from (1) the cell count per unit volume of the sample; (2) the number of staphylococci-producing colonies on agar from a unit volume prior to and following the rupture of the cells; (3) the frequency of infected cells

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* The stain used is of particular value in counting staphylococci within cells. The technic was developed by H. J. Baker and M. R. Ferguson by combining the crystal violet staining and differentiation of gram-positive organisms as described by Hucker[4] with a counterstain of Gomori's trichrome mixture.[5]

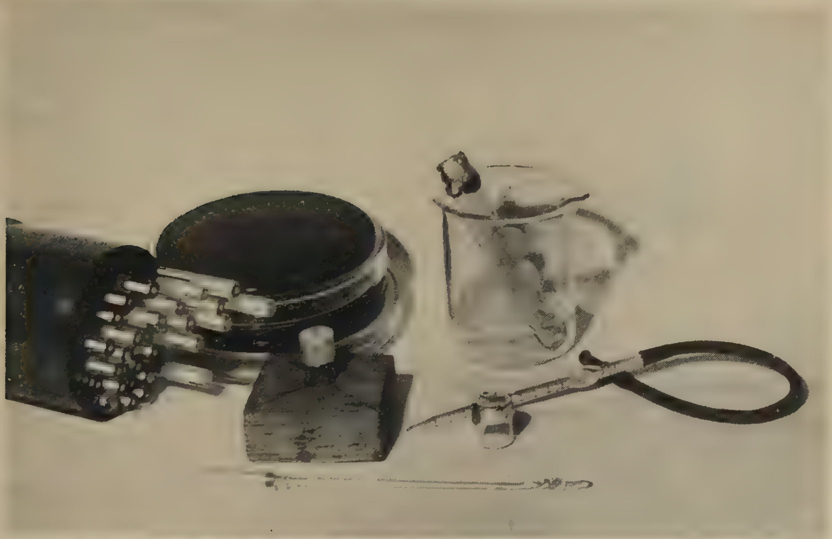

FIGURE 3. Materials for assay of infected cells. Rubber squeegee is shown within the segment of the cell tube after breaking off the tip. Tube for collecting the suspended cells, iced lustroid tube for vibration of sample, and shell vial containing round coverslip are shown.

among those centrifuged onto the coverslip; and (4) the numbers of staphylococci within the infected cells.

FIGURE 4 presents the results of studies of the stained coverslip preparation. The data have been accumulated by counting the total numbers of cells, including fragmented cells, the numbers of these cells which contain bacteria, the number of bacteria within these infected cells, and the number of extracellular cocci seen. Occasionally cocci were seen in a piece of material staining like cytoplasm, and these cocci were classed with those seen in fragmented cells.

The chart in FIGURE 4 shows that the number of infected cells and infected cell debris per 10,000 cells at "zero" hour is 17. This figure shows little change in the first two hours. At five hours there is an increase in this ratio to 24. The numbers of staphylococci in the infected cells, as demonstrated in the bar graphs of distribution, show no sign that the staphylococci have multiplied within the cells, and the total number of staphylococci seen in cells and debris show a definite decrease after "zero" hour. At the 5-hour interval there is an increase in the frequency of extracellular organisms. This frequency is believed to be related to the increased number of infected cells at the 5-hour period. The data on survival of viable organisms in these experiments are discussed later in conjunction with survival of organisms in streptomycin treated cells.

Survival of Staphylococci in Streptomycin Treated Cells

The same technic as previously described is used, and streptomycin is added to the nutrient media supplied the cells when flow starts at "zero" hour. The

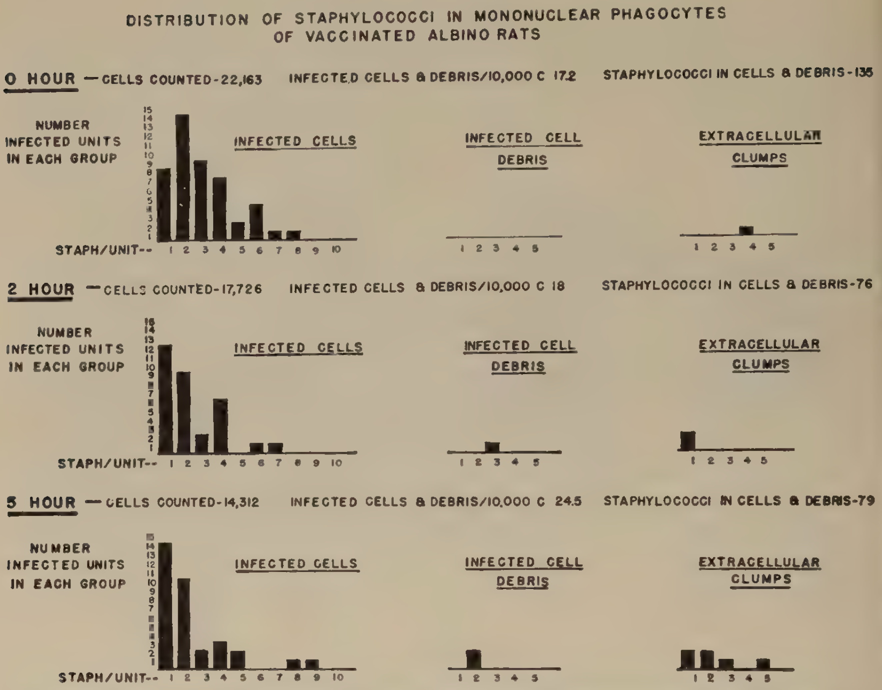

FIGURE 4. Data derived from counts on coverslip preparations of four experiments without the use of antibiotic. The source of cells was albino rats prepared with staphylococcus vaccine.

fluid used to suspend the cells for analysis is "bacto-penase" to provide for neutralization of remaining streptomycin activity. These cells were set up with the cells from rats vaccinated as before with formalinized staphylococcal vaccine and companion experiments were set up with cells from rats similarly vaccinated with a vaccine from a formalinized diphtheroid organism. This vaccine was shown to produce a residual of accumulated cells in regard to numbers and character like that of the staphylococcal vaccine. It was thought, in regard to signs of immunity, that the cells from nonspecifically immunized animals would behave more like normal cells. The concentration of streptomycin used in two trials on each set of cells was 1 μg./ml. and, in two trials, 10 μg./ml. There was no appreciable difference in the results caused by this degree of change in streptomycin concentration, and the results presented are compiled from four experiments with each of the two kinds of cells.

A measurement of cell-free viability of the staphylococci was provided in each experiment. Staphylococcal inocula corresponding to the number of viable organisms found at "zero" hour in the cell-culture experiments were put into 1-ml. nutrient culture medium containing streptomycin and incubated at 37° C. No viable organisms were recovered after two and one-half hours exposure to either 1 μg./ml. or 10 μg./ml. of streptomycin under these conditions.

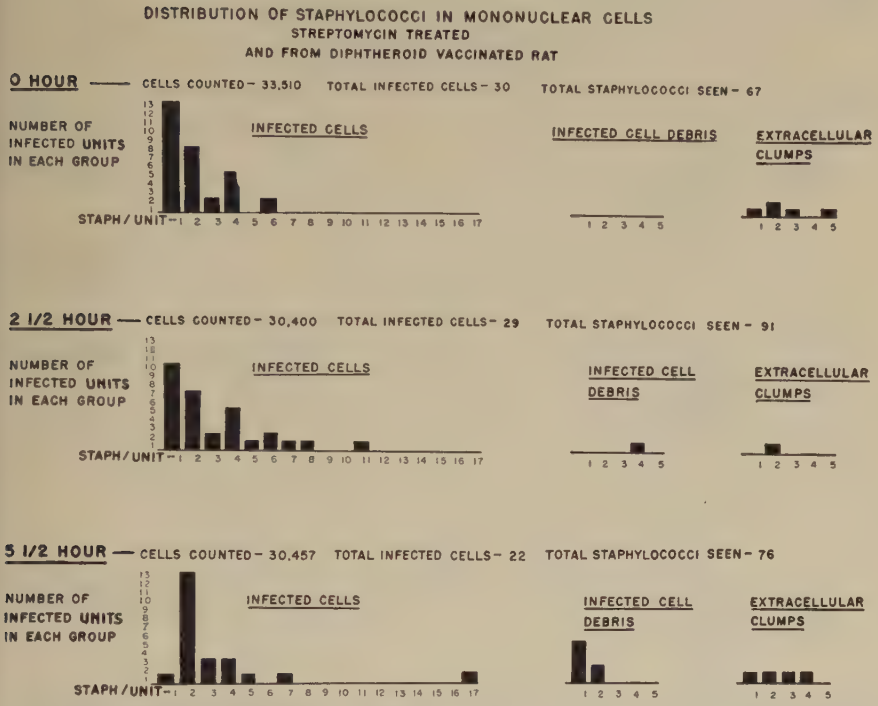

FIGURE 5. Data derived from counts on coverslip preparations of four experiments using streptomycin and cells from rats prepared with diphtheroid vaccine.

FIGURE 5 presents data obtained in the study of stained coverslip preparations of cells from the diphtheroid vaccinated rats treated with streptomycin. The relative number of infected cells at the outset is 30 per 33,510 cells, or approximately 9 per 10,000. At two and one-half hours the number of infected cells and infected cell debris is approximately 10 per 10,000 cells. At five and one-half hours the number of infected cells and cell debris is again approximately 10 per 10,000 cells. The presence of an agent that rapidly destroys extracellular organisms and prevents their accumulation in the extracellular fluid minimizes additional phagocytosis. The constancy of the ratio of infected cells in this series is an indication that cells lost in the flowing medium were lost in a random fashion with respect to infected and noninfected cells. The higher numbers of staphylococci in the infected cells shown by the extension of the distribution-curve at two and one-half hours is an indication of multiplication in these cells from the nonspecifically vaccinated animals, not seen in the cells from animals receiving the staphylococcus vaccine. At five and one-half hours the increased frequency of organisms in cellular debris is a reflection of injury to the infected cells of the culture.

FIGURE 6 presents data similarly obtained and compiled from four experiments with staphylococci in cells from rats receiving staphylococcus vaccine. These cultures were streptomycin treated as described above. In this set the

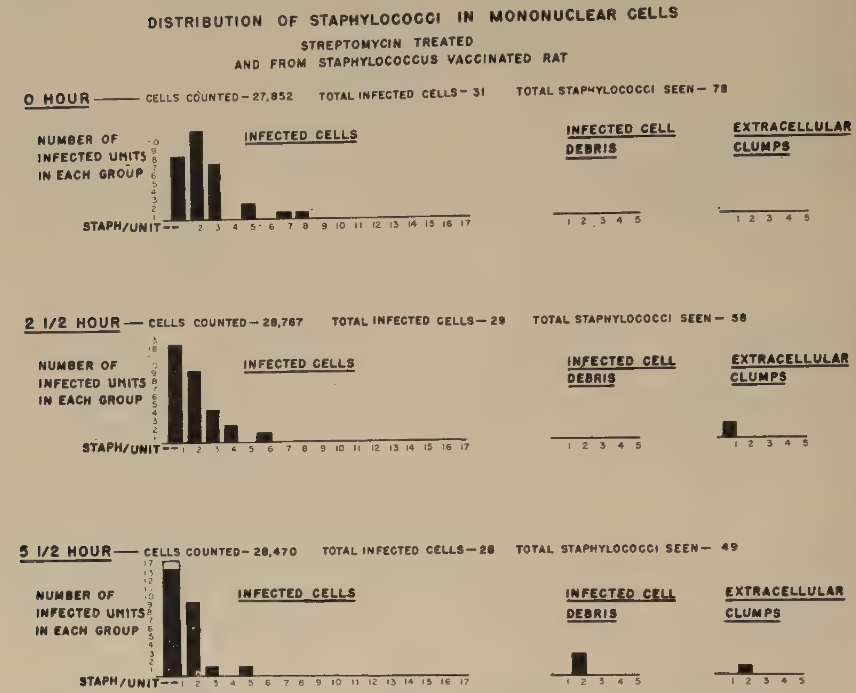

FIGURE 6. Data derived from counts on coverslip preparations of four experiments using streptomycin and cells from rats prepared with staphylococcus vaccine.

number of infected cells at "zero" hour is 31 in 27,852 cells, or approximately 11 per 10,000 cells. At two and one half-hours there are 29 infected cells in 28,767 cells counted or approximately 10 per 10,000 cells. At five and one-half hours, in 28,470 cells counted, the number of infected cells and infected cellular debris is 31 or approximately 11 per 10,000. The total number of staphylococci seen declines from 78 at "zero" hour to 58 at two and one-half hours to 49 at five and one-half hours. In observing the distribution of staphylococci in the infected cells it is noted that the number of infected cells showing three or more cocci decreases as the time progresses. This indicates the disappearance of bacteria

The disappearance of the bacteria cannot be considered as attributable to selective loss of infected cells during flow since the relative number of infected cells does not decrease. Either egestion of bacteria or intracellular destruction must be considered. If egestion of staphylococci were a constant feature in experiments described in this paper it should be most clearly manifest in experiments with nonspecifically immunized cells where the numbers of bacteria increased within the cells. For with the increasing numbers of organisms within the cells, there would be expected greater chances for egestion of bacteria, which should lead to rephagocytosis and an increased relative number of infected cells. This effect was not seen. Destruction of the bacteria does not entirely explain the loss of bacteria in the experiments with cells from the

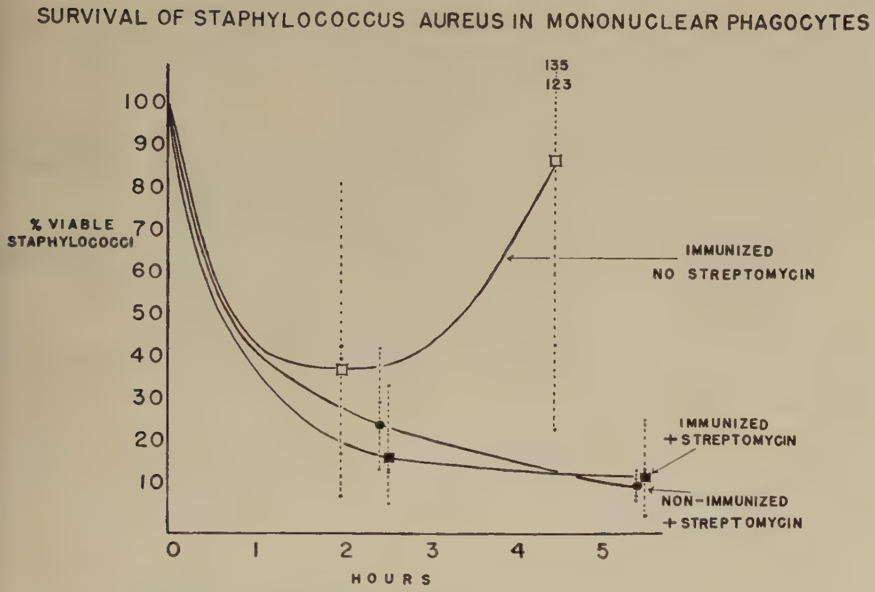

FIGURE 7. Trends of percentages of staphylococci found at "zero" hour surviving for 4½ to 5½ hours. The points on the vertical lines give the individual measurements and indicate the variation in the data.

staphylococcus vaccinated animal as there is no decrease in the relative number of infected cells. For the observations obtained, both mechanisms of the destruction of cocci and some redistribution of organisms among previously noninfected cells, must be used to explain the results.

FIGURE 7 represents the findings of percentage of viable staphylococci remaining after "zero" hour in the three groups of experiments with staphylococci that have been discussed. The numbers of viable staphylococci cultured is usually increased two or three times following disruption of the cells by sonic vibration. The highest count obtained in each case was the figure presented. There is wide variation in the compiled figures as indicated by the vertical lines on the graph. The solid lines of the graph pass through averages of these figures and indicate only the general course. The shape of the curves is an estimate from impressions of figures at one and four hours observed in other experiments on survival of ingested staphylococci in this laboratory.

The consistent depression of the percentage of viable organisms in the cells without streptomycin in the first two hours is a reflection of cellular activity. The consistent rise in percentage of viable organisms in this group from two to five hours, from considerations of data from the stained preparations, is thought to be related to the increase in extracellular organisms.

The mean depression at two hours in viable staphylococci from the organisms engulfed by mononuclear phagocytes of the rat receiving staphylococcus vaccine and not treated by streptomycin indicates that the depression seen at two and one-half hours in the number of viable staphylococci in cells from a similarly vaccinated animal and the streptomycin treated is in a considerable measure attributable to the activity of the cells. The marked difference of streptomycin treatment seen here is the elimination of the secondary rise in viable bacteria, which is thought to be largely attributable to the control of extracellular multiplication.

In those cells which came from animals not specifically vaccinated but streptomycin treated, in which there was previously some evidence of multiplication of bacteria, we see that the percentage of viable organisms remaining is not greatly different from those organisms residing in the cells of the immunized animal. This effect presents a marked contrast in data from the stained preparation, which shows an increase in the number of bacteria. Although there is indication of multiplication of organisms within the infected nonimmunized cells in the first two and one-half hours, destruction among these cells may lead to greater exposure of the organisms in these cells to streptomycin under the conditions of this experiment. The data of similar numbers of surviving organisms in the specifically and nonspecifically vaccinated cells in the presence of streptomycin cannot be interpreted to mean that there would be similar survival if transferred from an essentially two-dimensional cellular system to a three-dimensional system, as *in vivo*, where the destruction of cells results in abscesses. In abscesses the streptomycin exposure may be considerably reduced.

Survival of Staphylococci in Mononuclear Phagocyte Cultures Containing Penicillin Method

The method that has been employed in these studies differs from previously described experiments in that there is no flow. Suspensions of approximately 80 per cent mononuclear cells are obtained by rinsing the pleural space of normal rats by the described method with the exception that no bacteria are in the rinsing fluid. For each experiment six vials containing cells and staphylococci and six tubes containing staphylococci without cells are prepared. The cells are allowed to settle on the floor of a screw-capped vial 26 mm. in diameter, in which a coverslip 6 × 12 mm., No. 1 thickness, has been previously placed (FIGURE 8). After 30 minutes settling time all but 0.1 ml. of the overlying fluid is removed, and 0.2 ml. of BSS, containing approximately 300,000 staphylococci per ml. and 0.1 ml. rat serum, is added. Phagocytosis is then allowed to proceed for one hour at 37° C. in alveolar air in the shallow layer of fluid. Then 0.4 ml. BSS, 0.3 ml. rat serum and 0.1 ml. BSS containing 10 units/ml. penicillin, are added to give a final concentration of 1 unit/ml. penicillin, 40 per cent serum and 50 per cent BSS; 50 per cent of this medium is renewed after three days.

Approximately 300,000 staphylococci are incorporated in one ml. of this medium in each of six Pyrex tubes 12 × 75 mm. These tubes are incubated at 37° C. in alveolar air with the cell cultures. One such tube is sacrificed to measure for the number of surviving staphylococci at the same intervals as for the cell containing vials.

The vials are assayed at intervals of 3 hours, 20 hours, and at every 24 hour interval thereafter for six days. At the time of sacrifice of the vial the super-

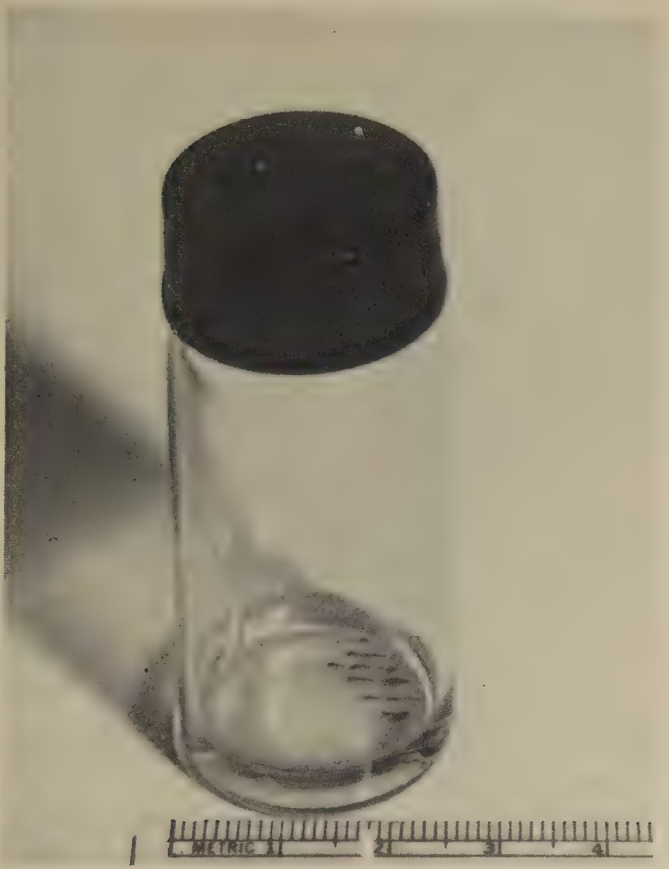

FIGURE 8. Culture vial for penicillin experiments with coverslip in place, marked for demonstration.

natant fluid is carefully removed and replaced by one ml. BSS containing 50 per cent "bacto-penase." The removed supernatant fluids are studied for cell content, viable staphylococci, and penicillin content. The assay of the vial contents is performed by first removing the coverslip for a strained preparation to count the relative numbers of infected cells, the number of cocci within the infected cells, and to observe the general relationship between cells and staphylococci. The cells remaining on the floor of the vial are suspended by gently wiping the surface with a sterile rubber policeman. From this suspension are obtained the aliquots for culture on blood agar before and after sonic vibration, and for a hemacytometer count of the number of cells in a volume of the suspension.

FIGURE 9 shows the survival of the staphylococci among mononuclear cells over a six-day period. The survival of similarly prepared and treated staphylococci without the cells is shown by the dashed line. Without cells almost all the bacteria are rendered nonviable by this concentration of penicillin in

24 hours. The staphylococci among the cells show a steady but gradual decline in viability through a period of five days.

The studies of the coverslip preparations showed no sign of multiplication of staphylococci within the cells after a three-hour period, when the first samples in these long-term experiments were taken. Many staphylococci could be seen within the cells in the three- and four-day periods when relatively few viable organisms could be found. A system in which a portion of the cell surface can be removed intact for staining gives information on the relations of the surviving organisms to the general population of cells. In the coverslip preparations from these experiments, the cocci are frequently located in loose foci of cells. Such arrangements give additional clues to the nature of the host-parasite relations, but they indicate that centrifuged suspensions on coverslips, as in the previously described technic, should be employed in addition, as such suspensions give a random distribution of the infected cells for counting purposes.

Summary and Conclusions

Techniques for study of bacteria in tissue culture are described. The results of experiments with staphylococci in rat mononuclear cells indicate that

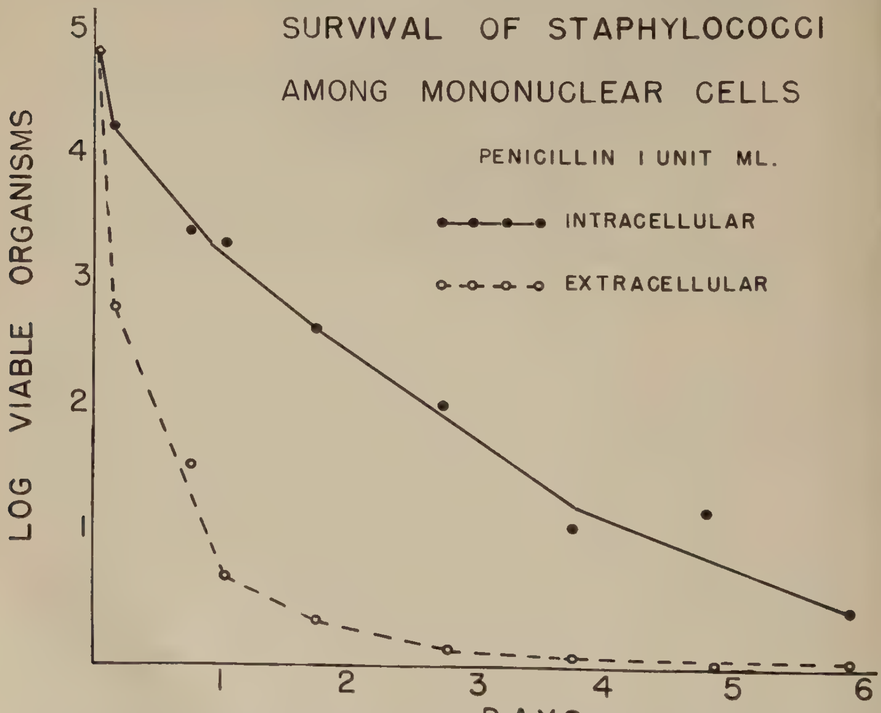

FIGURE 9. The survival of staphylococci is presented as the log of the absolute number of staphylococci found per vessel. The points represent the averages of the data from five experiments.

both multiplication and death may be occurring simultaneously in the population of intracellular microorganisms. Multiplication of staphylococci in cells from nonspecifically treated animals has been observed; however, in experiments with cells and with serum from immunized rats, no sign of multiplication of intracellular staphylococci is evident and, in these cases, even destruction of staphylococci has been noted. The techniques described present methods which may be used to elicit the relative influence of serum and of cells on the intracellular behavior of staphylococci. Such studies are in progress. Both penicillin and streptomycin show decreased activity on the organisms that are within the mononuclear phagocytes as compared to cell-free bacteria. Cellular activity is a major consideration in the elimination of staphylococci from these cultures.

The survival of staphylococci as individual organisms among rat mononuclear phagocytes treated with antibiotics is a multiphase dynamic process, and must be considered as such when tissue culture methods are used in attempts to gain some understanding of *in vivo* processes.

Acknowledgment

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ACTION OF VARIOUS SUBSTANCES ON THE MITOCHONDRIA OF LIVING CELLS CULTIVATED *IN VITRO*

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Several authors have attempted to modify, by the action of various physical or chemical agents, the structure or the enzymatic functions of mitochondria isolated by different techniques (centrifugation, dilaceration, *etc.*), or *in situ* (Anitschow,[1] Bensley,[2] Zollinger,[37] Harman and Feigelson,[23] *etc.*). It must be stressed that most of these researches were made on unfavorable material, or on isolated mitochondria. In the latter cases, the connections with other cellular elements with which the mitochondria have functional exchanges (Chévremont and Frederic,[22, 23] Frederic[16, 17]) were severed.

After having studied, with M. Chévremont, the chondriome of cells placed in normal conditions, it seemed to me interesting to follow, by means of phase microscopy and cinemicrography, directly on living cells, the behaviour of the chondriome under the influence of various substances of well-known properties.

First I investigated the action of *detergents* (wetting agents),[18] which concentrate at interfaces. Then I proposed to study a series of substances which could modify cellular metabolism. These researches are still in progress and, until now, I have centered my attention on *2.4 dinitrophenol*[19] and *phenylurethane.*

It is well known that nitro derivatives, among them 2.4 dinitrophenol, increase notably the metabolism in general and particularly cellular oxidations (Heymans *et al.*,[24, 25] Dodds and Pope,[12] Field-Martin and Field,[13] Chévremont,[5] *etc.*). The mechanism of action of dinitrophenol (DNP) has been the object of numerous investigations (*cf.* Summer and Myrback,[35] Krebs,[28] *etc.*); its toxicity in tissue cultures has been studied by Verne.[36]

On the other hand, phenylurethane, a narcotic, has been used in researches on metabolism and segmentation of frog eggs (Brachet[3], Hiller[26]); this substance adsorbs readily on the interfaces on which the cellular enzymes are fixed and inhibits dehydrogenases (Keilin[27] Quastel and Wheatley[33]).

Working on an identical cellular material, also at the Institute of Histology of the University of Liége, M. Chévremont and S. Chévremont-Comhaire, investigating the action of *Trihydroxy-N-methylindole* on growth, have shown its notable effect on the chondriome. These authors' results, which have been published in detail,[10, 11] will be quoted here and discussed in connection with our own findings.

Material and Techniques

Cultures (more than 4500 explants), in hanging drop, of fibroblasts and mononucleated myoblasts (chick embryo), following a technique previously used by us in researches on the chondriome.[8, 9, 21, 22]

Prolonged observations, serial photomicrographs, and cinemicrography in phase contrast of cultures maintained at 38° C.

Frame-by-frame analysis of the recorded films.[16, 20]

Vital staining with Janus green B, freshly prepared, at 1/10,000 or 1/20,000 in Ringer solution.

As a control some cultures were fixed and stained (Bensley liquid and iron haematoxylin).

Substances added:

Detergents: Sodium laurylsulfate, sodium laurylsulfonate, dodecylbenzene sulfonate, dibutylnaphtalene sulfonate, hexadecyltrimethylammonium bromide, aerosols OT and AY. Dilutions from 5 to 0.5 per cent in Ringer or Tyrode solution, or in Parker's glycosol.

2.4 dinitrophenol (sodium salt) freshly prepared stock solution at 2 per cent, diluted just before use in Tyrode solution; final concentration: 1/50,000 to 1/500,000.

Phenylurethane: 0.01 M stock solution in Ringer (isotonicity adjusted), diluted to final concentration of 0.005 M to 0.001 M.

Without any further specification, the cultures (after 24, 48, or 72 hours growth *in vitro*) are placed for one to five minutes in a solution of the substance to be studied; they are then mounted on special slides[7] for observation in phase contrast. In this way, only a thin layer of liquid covers the culture, which can still exchange gases with the atmosphere of the chamber. The control cultures are treated in the same way, but with Ringer solution only. In several cases, the investigated substances are added to the culture during the cinemicrographic recording by a special type of depressed slide, into which were fixed lateral tubes.

(*1*) *Effect of Detergents*

With the exception of dibutylnaphtaline sulfonate, inactive, all the detergents studied have a comparable effect. Their action shows two distinct phases: a first phase, during which the mitochondria filaments change into a string of beads and then fragment into grains; and a second phase, during which deep structural changes appear.

First phase. The first visible phenomenon is the appearance, in one or several places of the cellular periphery, of fine "spines," often in a group. Do these phenomena indicate the penetration of the detergent into the cell? After a variable but short time (in minutes) the filamentous mitochondria progressively lose their active movements, which, in most cases, cease completely. However, the viscosity of the cytoplasm is not modified, as shown by the fact that the movements of other cellular inclusions (fat droplets, vacuoles) in the cytoplasm remain unchanged.

During the slowing down of their active movements, the filamentous mitochondria take the aspect of a string of beads (FIGURES 1b and 2b), formed by a series of granules, short rods, or small spindles, in line on a very thin filament. These aspects are transitory at the start and parallel in several points with those often observed in normal intercinetic cells and constantly in cells during mitosis.[8, 9] Later this aspect is confirmed; suddenly, then, a liberation of the granules takes place (apparently by rupture of the thin filament on which the granules were lined). These grains become larger and their contrast increases

(FIGURE 2c). Only the filamentous mitochondria are modified; those found in the form of balls, observed for instance at the beginning of the growth *in vitro*, are not apparently modified.*

Second phase. After a period of rest, which can be very long, and only when the concentration of detergent is high enough, a second phase starts, which takes place very rapidly, even suddenly (about one minute). In the center of each grain formed during the first phase (apparently of intact ball-shaped mitochondria), a small grey area begins to appear; this area later clears up and enlarges rapidly. The mitochondrion is then formed of a clear central zone, with irregular grey bands and a black line border of irregular thickness (FIGURE 3a). The position of the grey bands and the thickness of the black line change continuously, as may be seen by the analysis of films. Eventually this instability of aspect stops and there is no further change. Often, however, a third phase follows the second. In such case the clear central area of each grain lights up strongly and the modified mitochondrion expands suddenly in a vesicle. It seems there is a dispersion by water intake (FIGURE 3b). During the second phase, signs of cellular death are observed: the nucleus, for instance, becomes more spherical and takes on a granular aspect, and its membrane seems to thicken.

(2) *Effect of Substances Modifying the Cellular Metabolism*

(a) *2.4 Dinitrophenol.* About 15 minutes after application of DNP (optimal doses: 1/100,000 to 1/200,000), irregular grey spots or marks appear in an area free of mitochondria, near the nucleus. These are often poorly contrasted and their limits badly visible.† A frame-by-frame analysis of the films gives evidence that these spots or marks are continually moving in the area and also changing their form and volume. Sometimes a mitochondrion penetrates into the area and seems to come in close contact with one of these grey marks. The surface of the "paranuclear zone" increases rather fast, then remains unchanged during a certain time, after which it diminishes and disappears some time after the other cellular modifications have reached their maximum.

During this same period, the *mobility*, the *aspect*, and the *volume* of the mitochondria are modified. First, the mobility is increased; the different kinds of active movements, which we have previously described[22, 23] in normal cells, become more intense and more rapid. This activation does not seem to be attributable to a modification of the physical state of the cytoplasm, for the movements of other cytoplasmic inclusions are not apparently modified. Later the mitochondria, still moving more actively than normally, tend to thicken; they become longer, by fusion of the ends of several short elements. The number of mitochondria seems to decrease in the cell. These modifications are particularly clear when the chondriome, at the beginning of the experiment, is represented only by short filaments (FIGURE 6). Under the action of DNP, the contacts between mitochondria and nucleus[22, 15, 18] become more frequent: in many cells, the nucleus is surrounded by long mitochondria coming in contact, at several points, with the nuclear membrane (FIGURE 6c), often where the nucleus is itself touching the other side of the membrane.

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* We should also note that, in some cases, another phenomenon can be seen. The cells are sometimes very thinly spreaded out and the mitochondria grouped around a small cytoplasmic area near the nucleus. When detergents are added, gray zones poorly contrasted and difficult to limit accurately appear in this area. It looks as if that region of the cytoplasm near the nucleus has a distinct structure, invisible in normal circumstances but having different reactive properties.

† On the films where the contrast is artificially increased, these bands can be more accurately analyzed.

Later, in most cells of the culture, the mitochondria tend to take another aspect: several of them stick together, thicken, and present local swellings which are sometimes very large (FIGURES 4b and c); this aspect is seldom seen in normal cells. Soon after, the "paranuclear zone" described above lessens in importance and, finally, disappears.

This evolution lasts between one and three hours without any sign of cellular degeneration and is reversible when the culture is washed in tepid glycosol. If the observation is continued, the chondriome can be seen to lessen perceptibly in volume, always keeping its abnormal form already described. The cell ends up by degenerating after several hours, a condition marked by the appearance of fat droplets, alteration of the nuclear membrane, and other changes.

A part of the experiments were made on cultures at the beginning of their growth *in vitro*, and at a moment when the chondriome is mostly in the form of small balls.[22, 23] Under the action of DNP the greater part of these balls are transformed into filamented mitochondria, in the same way as they do in normal cells, but much more rapidly. Several of them, however, disappear without being transformed into filaments, just as if they had "vanished" into the cytoplasm.

The nucleoli are also modified under the action of DNP. As shown by the analysis of films, their deformations are more rapid; they are more frequently in contact with the nuclear membrane, and they often move in the nucleus. It is possible to observe in them clear marginal or central zones of changing location and form, and a tendency to grow in size. Very often the cinemicrographic recordings show a progressive decrease in volume of one of the two nucleoli (FIGURE 5). With higher doses of DNP (1/40,000) the nucleolus is reduced to a group of small grains, or can be seen only with difficulty, and the cell finally degenerates.*

The age of the culture is very important for the intensity of the phenomenon. This evolution of the chondriome and the nucleoli is particularly clear in cells during the first and second day of growth. During the third day the cells have the same morphological aspect, but the doses of DNP producing the above described changes in younger cultures have no effect whatsoever.

(b) *Phenylurethane. A few minutes* after phenylurethane (optimal doses: 1/100 to 1/200 M) has been added to the cultures, the active deformations of mitochondria begin to slow down and finally stop almost completely. The displacements of mitochondria in the cytoplasm, however, and also of fine fat droplets which eventually appear, remain unchanged. In other words, the mitochondria, "fixed" in their form, are seen to be displaced passively in the cytoplasm. The viscosity of the fundamental substance does not seem to be modified, as far as can be seen. No other visible phenomenon occurs as regards the other cellular elements.

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* Apart from these actions on mitochondria and nucleoli, the mitosis is also affected. Whereas the divisions in normal cultures last between 90 and 120 minutes, DNP (with the same doses as those which activate on the chondriome) accelerates them to 45 minutes (on an average of 80 observations). This time is counted from the disappearance of the nuclear membrane to the complete reconstitution of the nucleoli in the two daughter cells. The increase of speed thus observed seems to be uniform on the different phases of mitosis. Moreover, mitoses considerably slow down or stop if cultures are observed at 30°C, and progress again normally under the action of DNP. More details on these observations will be published later.

After a few hours, movements and deformations of the mitochondria have again taken their normal characters. The mitochondria themselves are not apparently modified, either in aspect or in structure. If, after this moment, the culture is treated anew by phenylurethane, the mitochondria movements are stopped once more.

Twenty-four hours after treatment, in addition to the chondriome modifications to be described later, different kinds of abnormalities can be seen in many cells:

(a) First, the presence of several *cells having two or three nuclei* (FIGURES 7, 8, and 9). Such cells are rare in normal cultures. These nuclei, in the same cell, often differ clearly in size (FIGURE 9); nuclei without nucleoli can also be seen, but more rarely.

It is not yet possible, in the actual state of my investigations, to affirm that nuclei in these multinucleated cells appear following a process of direct division. Such a process, however, has been followed three times and in different cells, from start to finish. Furthermore, about 40 mitoses have been observed in these cultures, showing normal division of the cytoplasm. In cases where direct division has been observed, the evolution was comparable to that described by M. Chévremont[6] in muscular fibers cultivated *in vitro*; it lasts a long time (about 10 hours). An exceptional but interesting case was noted: after an attempt to direct division evolving up to an advanced stage, the nucleus returned to its normal aspect and then underwent normal mitosis with formation of two daughter cells.

(b) In the same cultures, many cells with *abnormally shaped nucleoli* can be seen; these nucleoli have irregular contours and a torn aspect (FIGURES 10 and 11). Sometimes the nucleus itself is also modified; it is larger than normal and has lost its habitual convex shape (FIGURE 11).

(c) Close to normal shaped cells, *very large cells* having voluminous nuclei can also be seen, but more rarely (FIGURE 12).

In many cells, principally in those having one or another of these abnormal aspects, the chondriome is modified. It has lost its normal form and is represented only by short, fine, poorly mobile mitochondria (FIGURE 12), sometimes grouped in little networks (FIGURE 8).

All the changes described above are irreversible; they happen even when the cultures are washed in tepid Ringer solution only a few minutes after their treatment by phenylurethane. Apparently it seems that phenylurethane has been adsorbed in a stable manner on cellular constituents, or that it initiated, in a short time, irreversible cellular modifications.

Supravital staining with Janus green B. As known, Janus green stains normal chondriome very electively. Whatever the mechanism of this staining, Janus green could be considered as an indicator of oxydoreduction.

Several lots of cultures, after 20 to 30 hours of growth, have been treated with phenylurethane (1/400 M), then stained by Janus green at different times after treatment.

In controls, mitochondria are electively stained; the other cellular elements remain colorless.

In treated cultures, results vary according to the time elapsed between treatment by phenylurethane and the vital staining:

Immediately, or a *few minutes* after treatment by phenylurethane, staining is no longer elective: mitochondria are stained, but cytoplasm and nucleoli also stain. The rest of the nucleus remains unstained.

Three hours after treatment (at a moment when, after a temporary stop, the movements of mitochondria are again normal), mitochondria are normally stained; the cytoplasm is again colorless, as it is in normal cells, but nucleoli remain stained. These abnormal aspects of the vital staining by Janus green will be discussed later. If the culture is treated anew by phenylurethane, staining reappears in the cytoplasm.

One day after treatment (when the form of the chondriome is modified and nuclei and nucleoli abnormalities are observed), the modified chondriome is stained but not so intensely as it is in cells where its normal aspect is guarded. The cytoplasm is almost unstained; the nucleoli are positive.

In other words, in cultures treated by phenylurethane, the supravital staining is unmodified in mitochondria, except when, after 24 hours treatment, are they modified in their aspect and are under the form of short and poorly mobile filaments. The coloration of cytoplasm appears clearly only when the time between treatment and vital staining has been short; if this time is too long (more than two hours), cytoplasmic coloration is almost imperceptible. In each case, the nucleoli are stained. These results will be discussed later.

Discussion

Before attempting to make general remarks on chondriome behaviour in the living cell, I shall discuss at some length the individual results obtained with the various agents.

In view of the interesting tendency of detergents to concentrate into interfaces, modifications of the chondriome under the action of these substances may be interpreted as follows:

During the *first phase*, the detergent, concentrating at the interface of the mitochondria, modifies the surface relations between them and the fundamental substance of the cytoplasm. Morphologically, filamentous mitochondria lose their active movements and take the form of a string of beads. The facts observed may be attributable to the direct effect of the detergent on mitochondria by modification of surface properties; dibutylnaphtalene sulfonate, which has a chemical structure similar to that of other investigated detergents but a poor surface-tension activity, has no effect on mitochondria. On the other hand, the observed evolution does not seem to be attributable to a secondary effect of cellular degeneration, since the other cellular elements have apparently not been altered during the whole of the first phase.

As to the *second phase*, the detergent acts as a denaturing agent once its concentration on the mitochondrion is attaining a threshold. The products of the denaturation of the lipoprotidic complex, of which the mitochondria are formed (Robertson,[34] Palade,[32,a] *etc.*), separate into two phases; one of these phases is formed on the surface of the other, looking like a "coat." This "coat" could have properties of a hemipermeable membrane; this potentiality could explain the sudden lighting up and the final bursting of the mitochondrion, as a dispersion by water. These pictures of the process are comparable to those obtained by other authors (Zollinger *et al.*, *etc.*), under the action of various agents, namely acetic acid after imbibition of distilled water by mitochondria. According to these authors, the "coat" surrounding the vesicle is a proof of the existence of a mitochondrial membrane. However, in contrast to this opinion, I think that the "coat" which was thus made visible, although it has, perhaps, osmotic properties, is an artifact and represents only one of the two phases resulting from denaturation of the mitochondrion.

The clear increase of active deformations and movements of mitochondria, under the action of dinitrophenol, is suggestive of an activation of cellular metabolism. Moreover, the increase in length of the mitochondria, the appearance of a "paranuclear zone" in a state of incessant motion, the more active deformations of nucleoli, and the more frequent contacts they have with nuclear membrane and mitochondria, are arguments which plead in the same direction. Although each of these phenomena can be observed separately in a few cells of a normal culture, their grouping in the same cell and in almost all the cells of the culture and their higher intensity, are characteristic.

But the activation of the cellular metabolism is probably not the only effect dinitrophenol has in these experiments. Several facts suggest that after the phase of activation, signs of alteration of metabolism (perhaps in the sense of a decrease of some syntheses) could appear, namely: the abnormal aspects mitochondria take after a certain time; the decrease in volume of the chondriome, which follows; the progressive disappearance of the "paranuclear zone" (admitting that it plays an effective role in the cellular metabolism); the pictures of nucleoli alterations, namely the decrease in volume often observed; and the appearance of clear areas.

These two aspects in the behaviour of the chondriome under the influence of DNP (first, a "phase of activation" followed then by a "phase of alteration"), correspond fairly well to the actual biochemical concepts concerning the mode of action of dinitrophenol; this substance increases noticeably the speed of respiration, but alters the metabolism by suppressing, in a specific way, the oxidative phosphorylations (*cf.* Summer and Myrbach,[35] Krebs,[25] *etc.*).

What is the meaning of the "paranuclear zone," which appears under the action of dinitrophenol? The fact it can be seen precisely at the moment when cellular metabolism is increased seems to indicate a not negligible role. Otherwise aspects that are similar but "motionless" become visible at the same place in cells treated by detergents. It can be supposed that it is a zone of particular structure normally invisible, but appearing when cellular exchanges are increased, or when its constituents are denatured by detergents. Is it the Golgi zone, or is it the cellular center? The question cannot be answered at present.

The first visible phenomenon, occurring a very short time after the culture has been treated by phenylurethane, is a progressive decrease, continuing to an almost complete stop, of the active deformations of mitochondria. Monné[31] has shown that ethylurethane, a substance closely related to phenylurethane, modifies the cytoplasmic aspect of a sea urchin's egg. In my investigations, however, the physical properties of the cytoplasm do not seem to have been modified, as far as can be seen. The fact that mitochondria, "fixed" in their deformations, are still passively moved in cytoplasm, as are other cytoplasmic inclusions (fat droplets, for instance), pleads in this direction. On the other hand, the cytoplasm of treated cells, as described above, is abnormally stained by Janus green. This effect could indicate an enzymatic alteration (Lazarow and Cooperstein[27]), more than a modification of the physical state; perhaps the dehydrogenases are implicated, as phenylurethane is known as an inhibitor of these enzymes (Keilin,[25] Quastel and Wheatley,[36] *etc.*). The pronounced slowing down of active movements of mitochondria, then, could be understood as the expression of an alteration of the cytoplasmic metabolism; this alteration is spontaneously reversible, and a few hours after the start of the experiment, the movements of mitochondria are again normal.

Without any further addition of phenylurethane, however, important morphological modifications appear after about 20 hours. It is noticeable that these modifications affect principally the nucleus and the nucleolus (multinucleated cells, irregular form of nucleoli, abnormal aspect of nucleus); they are irreversible, and happen even when the cultures are washed after their treatment by phenylurethane. It is possible that this substance alters the nuclear metabolism very quickly, either directly or indirectly, and in an irreversible manner. The fact that the nucleolus is stained by Janus green, from the beginning of the experiment, is abnormal and suggests a metabolic alteration.

This paper is not the place in which to discuss the genesis of multinucleated cells appearing in the cultures treated by phenylurethane. Probably the nuclei of these cells are formed by direct division, a phenomenon which has been followed from beginning to end; otherwise the mitoses observed in the same cultures give, each time, two daughter cells. In fact, Bucher,[4] investigating the effect of ethylurethane on mitosis of fibroblasts cultivated *in vitro*, never observed the absence of cytodieresis under the action of these substances.

M. Chévremont and S. Chévremont-Comhaire[9-11] have investigated the effect of *trihydroxy-N-methylindole* (THNI) on growth, in experimental conditions similar to mine and on an identical cellular material. THNI is an isomere of adrenochrome, and is supposed to block copper and zinc and to promote liberation of ascorbic acid in the organism. It is interesting to report here several of the results described by these investigators in order to compare them with mine. I shall then conclude by making a few general remarks.

Under the action of THNI, important modifications are observed in the chondriome. Mitochondria lose their mobility, and also are shortening, thickening, and becoming spherical. In certain conditions these phenomena are reversible. These mitochondria that are morphologically modified are altered in their constitution: among other changes, vital staining with Janus green and Baker's test for phosphoaminolipids become negative. A long time after the addition of THNI the total quantity of the chondriome is clearly increased. It appears that mitochondria are progressively modified and accumulate in the cytoplasm, and that new elements are formed at the same time. The substance, or one of its derivatives, has been detected in the cell itself, and seems to be localised in the cytoplasm, particularly on the chondriome.

From a general comparison of the results obtained with the investigated substances, the following considerations emerge. Whether mitochondrial movements are active or passive has been discussed for a long time (*cf.* namely Levi, 1934–1956). Many authors think that mitochondria are passively moved by cytoplasmic currents. In a recent publication, Zollinger still considers this opinion as the most probable. In a previous study, based on the observation of living normal cells under favorable conditions, M. Chèvremont and myself have proposed an hypothesis according to which these movements are not only passive but could be attributed to metabolic exchanges between mitochondria and cytoplasm. Modifications in the behaviour of the chondriome we have observed under the action of various substances give experimental arguments in favor of this hypothesis. Three of the investigated substances (detergents, THNI, phenylurethane) provoke, a short time after they have been added to the culture, a slowing down and a stoppage of the active movement of the mitochondria, the physical state of the cytoplasm appearing unchanged. These substances are precisely those which, by different mechanisms, alter the metabolic exchanges between mitochondria and cytoplasm. On the other hand, mitochondria deformations are clearly augmented under the action of dinitrophenol, a substance increasing the rate of respiratory exchanges.

Other aspects are interesting to compare: on the one hand, for example, the important accumulation of ball-shaped modified mitochondria, in cells treated for a long time by THNI; on the other hand, a progressive decrease in volume of the chondriome after a prolonged action of DNP. It seems that, in the first case, mitochondria accumulate because they continue to be formed while exchanges between mitochondria and cytoplasm are diminished or stopped; and that, in the second case, the chondriome is not replaced by the inhibition of certain syntheses. These facts are still difficult to interpret; they suggest that, in normal cells, there is an equilibrium between the cytoplasm and the chondriome, an equilibrium that can be experimentally unbalanced. These facts can also give some indication of the genesis of the chondriome. Until now the possibility of a "de novo" formation of mitochondria has been denied (Lansing,[30] Palade[32b]); however, the images observed in living cultures treated for a long time by THNI indicate that such a "synthesis" could occur.

Summary and Conclusions

The behaviour of the chondriome in living cells, under the action of various substances, has been studied in tissue cultures. Direct observation, serial photomicrographs, and cinemicrography in phase contrast are used for the

analysis. Substances investigated until now are: modifiers of surface-tension; *detergents*, modifiers of cell metabolism with a known action on certain enzymes; *2.4 dinitrophenol;* and *phenylurethane.* In most experiments, the active substances were added just before or at the beginning of observations or cine-recording.

Detergents (aerosols OT and AY, sodium laurylsulfonate, *etc.*) cause active deformations of mitochondria to stop progressively. This effect is probably attributable to the blocking of metabolic exchanges between mitochondria and the cytoplasm by a concentration of the detergent at the mitochondrion-cytoplasm interphase. Later, the mitochondria become beaded and are fragmented in small granules. If the concentration of the detergent is high enough, chemical constituents of the mitochondria are then denatured: they separate into two phases, one of which is placed at the surface of the other, as a "coat" and acting as a pseudomembrane with osmotic properties.

The *2.4 dinitrophenol* clearly stimulates active movements of the mitochondria, which progressively become longer and thicker. Nucleoli are more actively deformed, and the contacts between nucleus and chondriome are more frequently observed. These pictures seem to indicate an activation of cellular metabolism. Different facts, however, suggest that after this "phase of activation" signs of metabolic alterations (perhaps a decrease of certain syntheses) appear: the mitochondria take on an abnormal aspect, the nucleoli shrink and have a "porous" aspect; and the chondriome also decreases in volume after a certain time. These modifications are generally reversible.

Under the action of phenylurethane, the active deformations of mitochondria are strongly slowed down. As far as can be seen, however, the physical state of the cytoplasm has not been modified; on the other hand, the fact that it is stained by Janus green, an abnormal aspect, indicates a metabolic alteration. It is possible that this change is responsible for the stoppage of the mitochondrial movements. These phenomena are spontaneously reversible. However, without further addition of phenylurethane, important morphological modifications appear after about 20 hours. These modifications principally affect the nucleus and the nucleolus, resulting in the appearance of multinucleated cells, probably produced by direct division; irregular forms of nucleoli; and abnormal aspect of the nucleus. All these changes are irreversible. In many cells, principally in those showing one or another of these abnormal aspects, the chondriome is represented by short and poorly mobile mitochondria. Different facts suggest that phenylurethane alters the nuclear metabolism in a very short time.

Some general conclusions may be drawn from these results and from a comparison of them with results obtained by M. and S. Chévremont, who investigated the effects of trihydroxy-N-methylindole. The observed mitochondrial movements and their modifications under the action of various agents favor the hypothesis that these movements are attributable to metabolic exchanges between mitochondria and cytoplasm. Different facts suggest that there is an equilibrium between chondriome and cytoplasm in living cells, and that a "de novo" formation of mitochondria can occur.

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Discussion of the Paper

Doctor F. Wassermann (*Argonne National Laboratory, Lemont, Ill.*): Doctor Frederic considered the possibility of the new formation of mitochondria. Concerning this point I should like to say a few words about my own findings regarding the mitochondria in the cells of pea-germs in the beginning of germination. Shortly after the moistening of the dried germs, when fixation and embedding, *etc.* become possible, no mitochondria were found in the cells of the embryonic root. But within the next 12 to 24 hours a few acid fuchsin stained plump rods and granules appeared in the immediate vicinity of the nucleus. The material was often found located in indentations of the nuclear membrane. Somewhat later typical mitochondria in increasing numbers were present over the entire cytoplasm. These findings suggested that in the pea-germ the mitochondria are not preserved during the period of dehydration and are reformed with participation of nuclear substances in the beginning of germination. Part of the illustrations were reproduced in G. Hertwig's article in von Moellendorff's *Handbuch der mikroskopischen Antomie*, Vol. I, 1st part. Berlin, 1929.

PLATES

Plate 1

Action of Detergents

Living cells observed at 38°C. by phase contrast microscopy.

First phase:

Figure 1. Two photomicrographs of the same cell in a culture treated by hexadecyltrimethylammonium (magnified 1400 times after enlargement). (*a*) Three minutes after addition of the detergent. A long filamentous mitochondrion, of normal aspect, twining around the nucleus. (*b*) Eighteen minutes later. The mitochondrion has broken into granules, still united in several places by a very fine and hardly visible filament (for instance near the right side of the nucleus). Nucleus, nucleolus, cytoplasm have kept a normal aspect.

Figure 2. Three pictures enlarged from a cinemicrographic recording (in 16 mm.) at very high magnification and showing the evolution of a filamentous mitochondrion under the action of aerosol OT (0.1%). Final magnification 2400 times. (*a*) Aspect of the mitochondrion at the start of the experiment. (*b*) Four minutes later. Fragmentation into grains; in several places, the grains are still united by a very fine filament. (*c*) Seven minutes later. Several of the grains have fused to form highly contrasted balls.

Second phase:

Figure 3. Two photographs (enlarged from a 16 mm. film; final magnification 1800 times) of a cell treated by aerosol OT (0.1%). (*a*) Twelve minutes after addition of the detergent. Beginning of the second phase: in the center of each grain formed during the first phase, there appears a clear central area. This area is surrounded by a black line of irregular thickness. (*b*) Two minutes later. The clear central area of the each grain has suddenly swollen and the grains have burst. The black surrounding band has condensed at one of the poles of the vesicle thus formed.

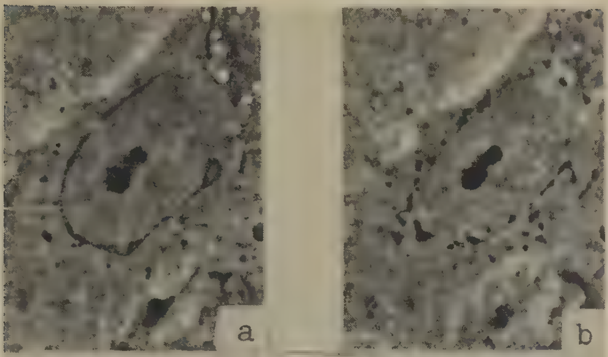

FIG. 1.

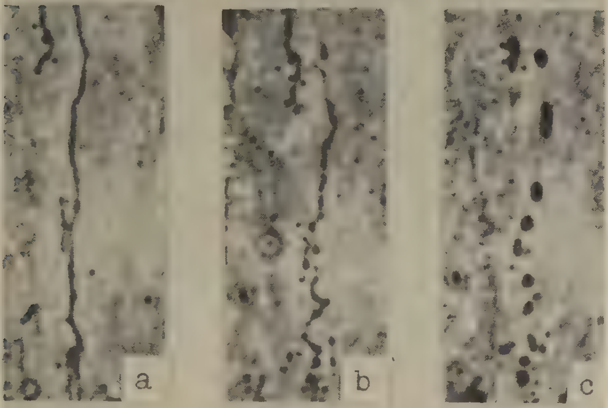

FIG. 2.

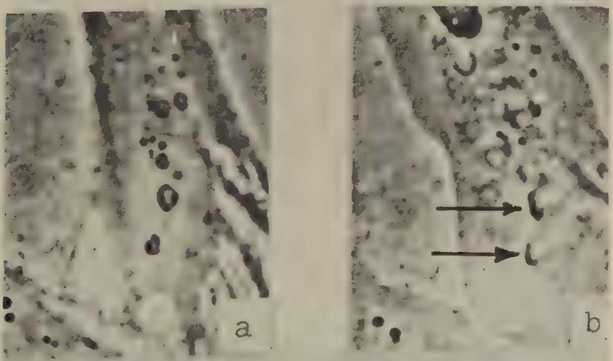

FIG. 3.

PLATE 2

Effect of the 2.4 Dinitrophenol

Living cells studied at 38°C. by phase contrast. Enlargements of pictures taken from 16 mm. films (final magnification 1,800 times).

FIGURE 4. The same cell photographed at different periods of time in a culture treated with dinitrophenol (1/150,000). (*a*) Thirty-five minutes after the addition of DNP. The chondriome, which became more abundant under the action of DNP, is found in the form of numerous filaments. The little black grains are fine fat droplets. (At the base of the picture, a part of the nucleus and of one nucleolus is visible.) (*b*) and (*c*) Respectively 100 and 120 minutes after addition of DNP. The number of mitochondria has decreased in the cell, several have united to form elements of greater thickness and often to acquire a very characteristic aspect (irregularity of form, with important local swellings). Note the paranuclear zone (groups of irregular grey bands, near the nucleus) and also the "porous" aspect of the nucleoli.

FIGURE 5. Two pictures of a cell in a culture treated with DNP (1/150,000) after only 20 hours of growth. (*a*) At the beginning of the experiment. Above and left of the nucleus, a group of mitochondria in balls, a typical feature of the cells after only a few hours of growth. (*b*) Two hours after addition of DNP. Only a few ball-shaped mitochondria remain. The others have rapidly transformed into filamentous mitochondria (see the large mitochondria in the upper part of the picture). Note the important decrease in volume of one of the nucleoli, in contact with the nuclear membrane.

FIGURE 6. A cell at different periods of time; it was chosen for the experiment because its chondriome was less abundant than usual. Under the action of DNP (1/200,000) the chondriome increases in volume; it first seems to recuperate back to normal, then to be more activated. (*a*) Ten minutes after addition of DNP. The mitochondria are still fairly short and fine filaments. The paranuclear zone (at the center of the picture, near the nucleus) begins to appear. (*b*) Twenty minutes later than in (*a*). Several short mitochondria have grouped together, forming longer elements. The aspect of the chondriome, at this stage of its evolution, is the same as in normal cells. (*c*) One hour later than in (*b*). Exaggeration of the normal aspect. The filamentous mitochondria are now clearly thicker and have become much longer. The greater part of them are grouped around the nucleus and are in contact at several places with the nuclear membrane. The paranuclear zone is clearly visible just below the nucleus.

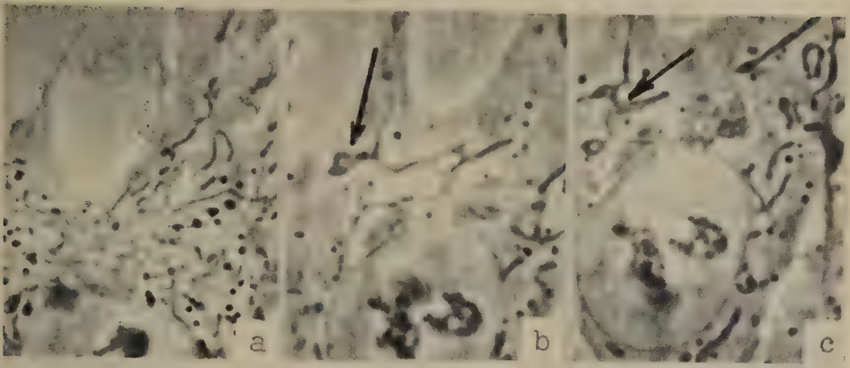

FIG. 4.

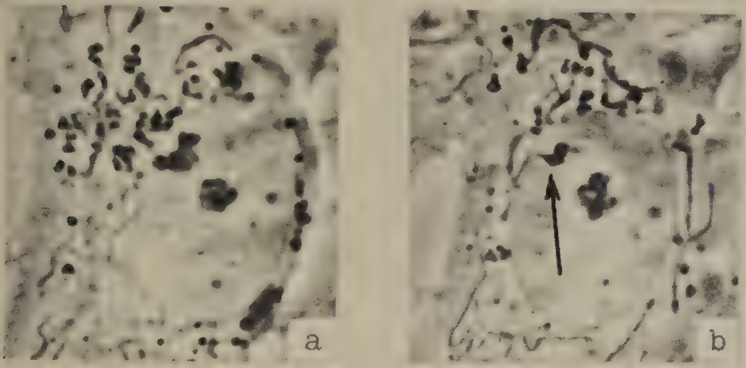

FIG. 5.

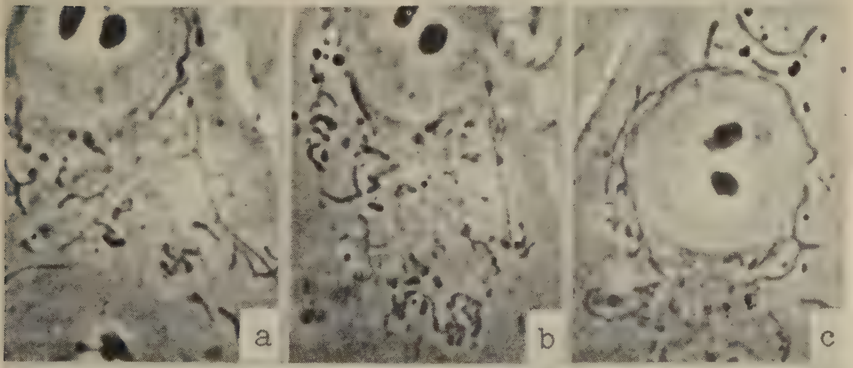

FIG. 6.

PLATE 3
Action of Phenylurethane

Photomicrographs of fixed and stained cultures (Bensley liquid-iron haematoxylin, magnification 1200 times). In the cells of FIGURES 7, 9, and 10, differentiation has been important and the chondriome is not stained.

FIGURE 7. Binucleated cell in a culture treated for 24 hours by phenylurethane (1/500 M). In one of the nuclei (the nucleus just below the center of the image), the nucleolus tend to take an abnormal aspect. The black traces in the cytoplasm are fat droplets.

FIGURE 8. In this cell (phenylurethane 1/600 M for 24 hours), direct division of the nucleus has been observed until it has reached the stage seen in the picture. The two nuclei thus formed are still in contact but clearly limited; note their different shape. In the upper part of the cell, the chondriome forms small networks.

FIGURE 9. A trinucleated cell in a culture treated by phenylurethane (1/400 M) from explantation. Note the difference in size of the nuclei. (The black mark on top is an accumulation of fat droplets.)

FIGURE 10. Phenylurethane 1/600 M. Irregularly shaped nucleoli in a cell treated 20 hours previously. One of these nucleoli is in contact with the nuclear membrane (upper part of the nucleus).

FIGURE 11. A nucleus of clearly abnormal shape (culture fixed one day after treatment by phenylurethane). In the nucleus two nucleoli, quite distinct in shape, are both in contact with the nuclear membrane; one of them is very irregular.

FIGURE 12. A very large cell in the same culture as in FIGURE 10 and at the same magnification. The nucleus is also larger than normal, it has lost the regular spheroid or round form habitual in the normal cell. The mitochondria are short and rather numerous. As observed in phase contrast before fixation of the cell they were not very mobile.

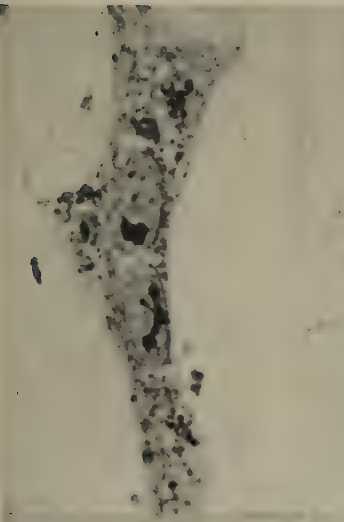

FIG. 7.

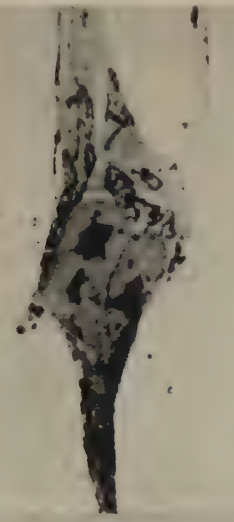

FIG. 8.

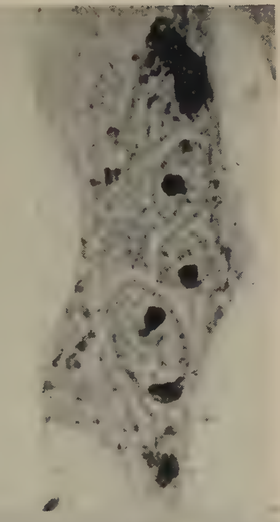

FIG. 9.

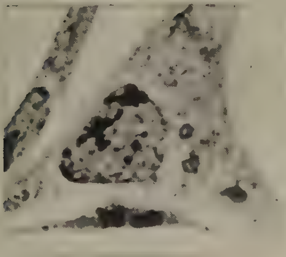

FIG. 10.

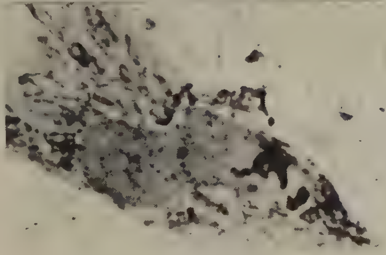

FIG. 11.

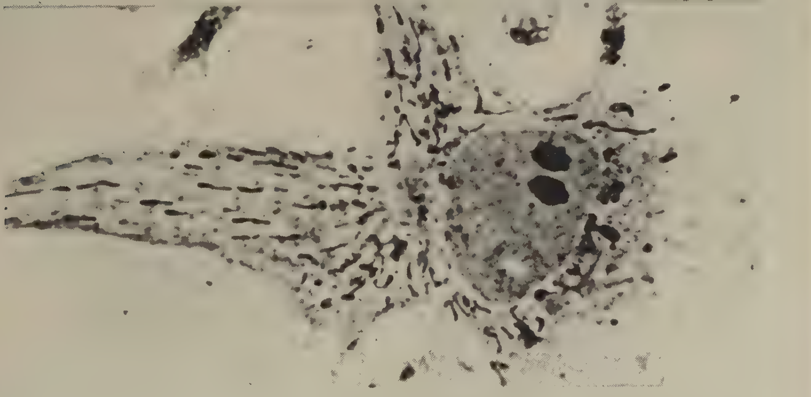

FIG. 12.

SYNERGISTS AND ANTAGONISTS OF MITOTIC POISONS

By Hans Lettré
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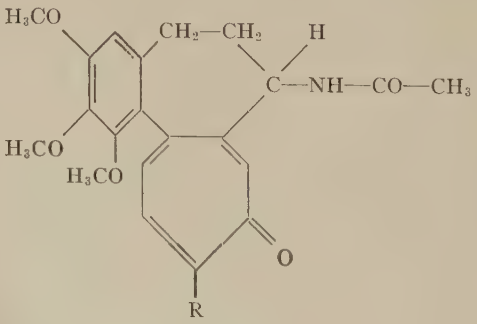
In 1939 we started to investigate the actions of single chemical compounds on cells grown *in vitro.* In particular, those compounds which show an antimitotic activity attracted our interest. Since 1945 we have carried out experiments involving combinations of a mitotic poison with one or more substances which do not possess this property. The results we obtained appear to be relevant for an interpretation of the action of mitotic poisons, as well as for an understanding of the rôle played by the medium in that process.

Methods

The technic[1,4] employed was as follows: a fibroblast culture from the mesenchymal tissue of the chicken embryo was transferred 15 to 20 times in order to assure its uniformity for the experiments. The explants were grown by the hanging-drop method. One half of a 48-hours-old culture was used as a control in a medium containing one drop of chicken plasma, one drop of embryonic extract, and one drop of saline solution. The other half of the explant was cultivated in the same medium: the saline solution, however, contained the compound to be tested. After 20 hours, a series of such control explants and experimental explants were compared under the phase-contrast microscope. Differences between the treated and the nontreated explants were recorded. Twenty-four hours after the explantation (in some experiments 48 hours afterward) the explants were fixed and stained, and their mitotic indices were determined. Other morphological changes were also noted.[2] Every day, a stock of 200 to 300 cultures of such a strain must be available in order to select the 20 to 30 best ones for experimental purposes.



Ia R = —OCH₃ Colchicine
Ib R = —NH·CH₃ N-methylcolchicamide

The addition of a mitotic poison to a tissue culture causes an arrestment of cell division. The degree of the arrestment depends on the amount of the mitotic poison added. The results produced by the addition of a mitotic

TABLE 1

Added dosage in μg./ml.		Sum is equivalent to		Percentage of mitoses	Spindle + = intact − = damaged
Colchicine	N-methyl-colchicamide	Colchicine	NMC		
—	—	—	—	1.9	+
0.02	—	0.02	0.0057	27.0	−
0.016	—	0.016	0.0045	16.0	−
0.01	—	0.01	0.0028	5.5	(+)
0.008	—	0.008	0.0023	3.5	+
0.0066	—	0.0066	0.0019	2.2	+
0.005	—	0.005	0.0014	1.5	+
—	0.008	0.028	0.008	40.6	−
—	0.004	0.014	0.004	10.0	−
—	0.002	0.007	0.002	3.9	−
—	0.001	0.0035	0.001	2.6	+
0.008	0.001	0.0115	0.0033	7.5	+
0.008	0.004	0.022	0.0063	37.6	−
0.01	0.001	0.0135	0.0038	6.4	−
0.01	0.002	0.017	0.0048	15.6	−
0.01	0.004	0.024	0.0068	36.5	−
0.005	0.002	0.012	0.0034	3.6	(+)
0.005	0.004	0.019	0.0054	5.5	(+)
0.02	0.002	0.027	0.0077	40.2	−
0.02	0.004	0.034	0.0097	61.4	−

poison such as colchicine (Formula Ia) is shown in TABLE 1. This table also demonstrates the results obtained with N-methylcolchicamide (NMC), according to Formula Ib.[3,4] We have tested mitotic poisons as well as other compounds by means of this method.[5] The results are reproducible provided the same strains of cells are employed.

The addition of a second compound to the medium of a tissue culture which already contains a mitotic poison may have no influence at all, or it may enhance (as a synergist) or diminish (as an antagonist) the activity of the mitotic poison. If, from the chemical point of view, the second compound represents a closely related mitotic poison, the mixture of both will result in an additive action, *e.g.* colchicine + NMC (TABLE 1).

Narcotine is a weak mitotic poison[6] since a concentration of more than 20 μg./ml. is necessary to produce an antimitotic effect (20 μg. 7 per cent, 40 μg. 11 per cent, control 2 per cent mitotic index). The addition of narcotine to NMC, however, reinforces the effect of NMC (*cf.* CHART 1).[7] Because of its ability to increase the antimitotic activity of a threshold-dosage of NMC it seems obvious that narcotine exerts some kind of influence on the cell other than is recognizable by morphological inspection.

Compounds without morphologically recognizable effects, however, may act as synergists also. TABLE 2 summarizes the results obtained by a mixture of the alkaloid bulbocapnine with colchicine.[4] Bulbocapnine alone did not affect cell division in concentrations as high as 80 μg./ml., while 4 μg./ml. of it produced a marked increase of the antimitotic action of colchicine.

The synergistic effect is not caused simply by any toxic compound but is dependent on the chemical structure of the synergist. We have undertaken, therefore, a comparative study of 10 chemically related compounds of the aporphine group, of which bulbocapnine is a member.[8] TABLE 3 shows the different compounds of that group which were tested. Only 5 out of 10 compounds did act as synergists. The specificity of the synergistic action is demonstrated by the close chemical relationship of both the active and inactive compounds.

Phlorhizin, an inhibitor of phosphatases, is another example of a synergist which we have tested.[9] Phlorhizin alone has no antimitotic activity but can produce an effect with a dosage of colchicine (*cf.* TABLE 4) which is too low to be effective by itself (*cf.* TABLE 4). In its ability to activate an underthreshold dosage of colchicine, phlorhizin differs from the synergists previously mentioned, *e.g.* bulbocapnine, which needs at least a threshold dosage of colchicine to be effective. This difference is important for the classification of the synergists.

The results can be interpreted by assuming an analogy between muscle contraction and the contraction of cellular elements. The existence of such an analogy has been postulated by morphologists,[10] physiologists,[11] and cytochemists.[12] In this concept, adenosinetriphosphate (ATP) and—via the intermediary metabolism—other compounds such as creatinephosphate, coenzyme A, and acetylcholine are connected with the contraction of cellular elements.

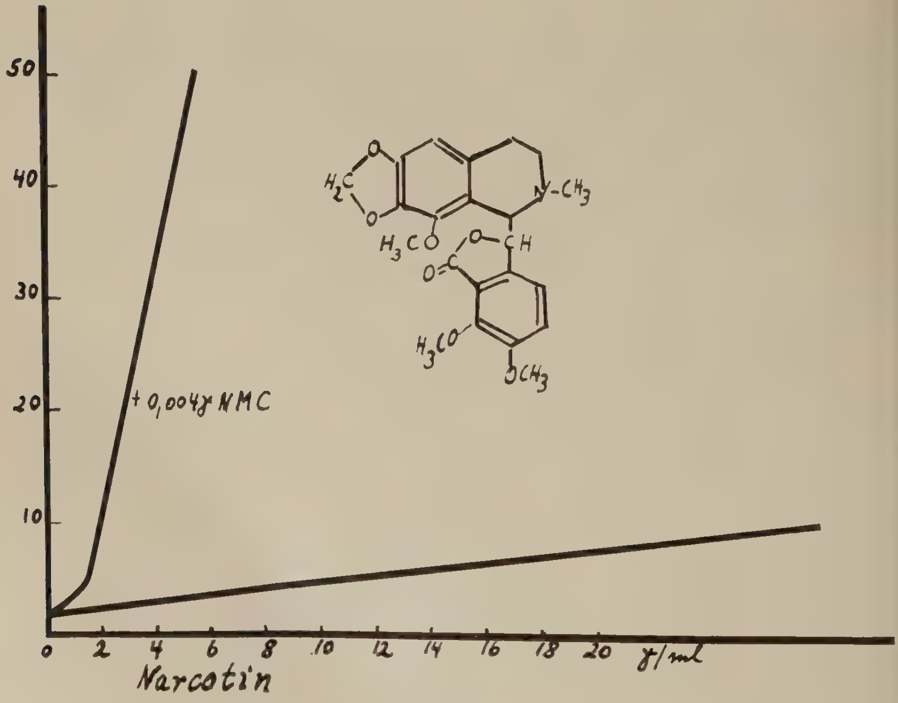


CHART 1

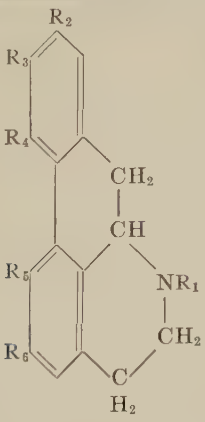
TABLE 2

Added dosage in μg./ml.		Percentage of mitoses	Spindle
Colchicine	Bulbocapnine		
0.005	8	1.1	+
0.0055	8	1.7	+
0.0066	8	2.3	+
0.008	8	24.4	−
0.008	4	19.3	−
0.008	2	3.4	(+) +
0.008	1	3.2	+
0.008	0	3.5	+
0.01	4	24.4	−
0.01	5	23.8	−
0.01	10	25.2	−
0.01	20	56	−
0.02	8	46.6	−
0.02	10	71	−
0.02	20	84.4	−
0.02	40	78.3	−

TABLE 3

+ = synergist
− = no synergist

	R$_1$	*R*$_2$	*R*$_3$	*R*$_4$	*R*$_5$	*R*$_6$	Effect
Apomorphine	—CH_3	—H	—OH	—OH	—H	—H	−
Morphothebaine	—CH_3	—H	—OCH_3	—OH	—H	—OH	−
Corytuberine	—CH_3	—H	—OH	—OCH_3	—OH	—OCH_3	−
Corydine	—CH_3	—H	—OCH_3	—OCH_3	—OH	—OCH_3	+
Bulbocapnine	—CH_3	—H	—OCH_3	—OH	—O—CH_2	—O—	+
Boldine	—CH_3	—OH	—OCH_3	—H	—OCH_3	—OH	−
Glaucine	—CH_3	—OCH_3	—OCH_3	—H	—OCH_3	—OCH_3	+
Laurotetanine	—H	—OH	—OCH_3	—H	—OCH_3	—OCH_3	+
Laurotetanine methylether	—H	—OCH_3	—OCH_3	—H	—OCH_3	—OCH_3	+
N-Acetyl-laurotetanine	—$COCH_3$	—OH	—OCH_3	—H	—OCH_3	—OCH_3	−



Aporphine-Derivatives

TABLE 4

ACTION OF PHLORHIZIN WITH AND WITHOUT ADDITION OF COLCHICINE

Added dosage in μg./ml.		Percentage of mitoses	Spindle
Phlorhizin	Colchicine		
—	—	1.9	+
375	—	2.0	+
188	—	2.0	+
94	—	1.8	+
47	—	1.6	+
—	0.011	6.4	+ (+)
188	0.011	80	−
94	0.011	47.2	−
47	0.011	39.5	−
23.5	0.011	36.6	−
12	0.011	11.8	−
6	0.011	16.9	−
3	0.011	12.3	−
1.5	0.011	6.6	+ (+)
—	0.0055	2.2	+
188	0.0055	35.4	−
94	0.0055	13.7	−
—	0.0045	2.6	+
94	0.0045	5.3	+
12	0.0045	4.0	+
—	0.003	2.4	+
94	0.003	4.6	+
12	0.003	3.6	+

Besides being upheld by the antagonistic action of ATP toward colchicine,[5, 13] this concept was further supported by our observation of the antagonistic effect of creatinephosphate toward some of the synergists. Creatinephosphate counteracts the synergistic effect of veratrine[14] but does not show antagonistic action toward colchicine (*cf.* TABLE 5). Barany and Palis[15] were able to demonstrate that colchicine influences the decrease in viscosity of a mixture of actomyosine and ATP. These results lend further support to our assumption that these factors are related to contraction of the spindle. H. H.

TABLE 5

Added dosage in μg./ml.			Percentage of mitoses	Spindle
Veratrine	Colchicine	Phosphagen		
—	—	—	2.0	+
—	—	600	2.0	+
—	0.011	—	4.0	+
—	0.011	600	7.6	+
—	0.022	1200	22.0	−
40	—	—	2.8	+
20	—	—	2.8	+
10	—	—	2.6	+
10	0.011	—	21.0	−
5	0.011	—	9.6	−
10	0.011	600	6.0	+
5	0.011	600	3.8	+

Weber and Hoffmann-Berling[16, 17] found that cells extracted with water and glycerol are induced to contract by the addition of ATP. In these cells the spindle, if present, will also perform contractions after the addition of ATP. Our observations on the contraction of the cell surface[18, 19, 20] are in agreement with the results of Goldacre and Lorch,[21] who concluded that alterations in the shape of amoebae are caused by partial contraction of the cell surface. At present, the results of Weber do not allow definite conclusions as to the behavior of living cells. The reslackening which Weber observed on his models was caused by the addition of very high dosages of ATP (10×10^{-3} M). Such a reaction has not been found to occur in muscle or tumor tissue. Le Page[22] estimated the maximum ATP-content of muscle and tumor with 5.4×10^{-3} M and 2×10^{-3} M respectively. We were able to note only a rigidity of the cell on adding 10^{-2} M ATP, which was reversible as the ATP was being degraded in the medium (*cf.* drug action on cells *in vitro*; demonstration of a motion picture by Doctor Renate Lettré).

The results of our studies on synergists may be interpreted by assuming either a disorder of the ATP-synthesizing metabolic processes or a direct influence of these compounds on contractile elements of the cell such as the spindle and the cell surface. Most of the synergists are also muscle poisons.[9, 23]

There are other compounds which may act as synergists, though their relation to the mechanism of contraction is as yet unknown. In this respect the steroid hormones are of especial interest. The hormones were added in crystalline form in order to exclude secondary effects caused by the solvent. TABLE 6 shows the synergistic effects of steroid hormones[24] on colchicine. Testosterone reinforces the activity of a threshold dosage of colchicine, while estrone

TABLE 6

SYNERGISTIC EFFECTS OF STEROID HORMONES ON COLCHICINE IN TISSUE CULTURES

Hormone*	Colchicine (μg./ml.)	Percentage of mitoses
—	0.01	5.0
Testosterone	—	2.4
Testosterone	0.01	21.0
Testosterone propionate	—	2.6
Testosterone propionate	0.01	13.0
Estrone	—	2.6
Estrone	0.01	3.4
—	0.016	14.2
Estrone	0.016	21.3
—	0.024	42.5
Estrone	0.024	62.2
—	0.005	2.0
—	0.0075	2.5
—	0.010	5.5
Desoxycorticosterone acetate	0.005	1.2
Desoxycorticosterone acetate	0.0075	2.0
Desoxycorticosterone acetate	0.010	9.0
Cortisone	0.005	3.3
Cortisone	0.0075	8.2
Cortisone	0.010	31.3

* Hormones added in solid form to the medium of the tissue culture.

TABLE 7

— = no synergist
(+) = synergist with overthreshold dosage of colchicine
+ = synergist with threshold dosage

Cholesterol	—	Androstenediol-3-acetate	+
Cholesterolacetate	—	Androstenediol-3-acetate-17-benzoate	—
Cholesteroloxyde	—	Epidehydroandrosterone	+
Cholestantriol	—	Epidehydroandrosterone acetate	+
Estrone	(+)	Pregnenolone	(+)
Equilin	(+)	Progesterone	(+)
Equilinglycol	(+)	Desoxycorticosterone acetate	+
Testosterone	+	Cortisone	+
Testosterone-propionate	+	Cortisoneacetate	+
Testosterone-benzoate	—	Isocortisoneacetate	(+)

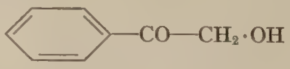
requires an overthreshold dosage in order to be active as a synergist. Adrenal cortical hormones, both desoxycorticosterone acetate (DOCA) and cortisone[25] (*cf.* TABLE 6) are synergists of colchicine. Unlike DOCA, cortisone is able to make effective an underthreshold dosage of colchicine.[26] TABLE 7 summarizes a number of steroids and derivatives which were tested,[27] demonstrating again the dependence of the synergistic action upon chemical structure.

On the basis of the results of the synergistic effects of adrenal cortical hormones, we tested the action of synthetic ketoles. Acetole was effective as a weak synergist in a concentration of 100 μg./ml., while phenylketol and phenylacetole, derivatives of phenylacetole, and stilbane-ketoles (Formula II-V) reinforced the antimitotic activity of colchicine in concentrations of 50, 10–25, and 1 μg./ml. respectively. The stilbane-ketoles were effective in the same concentrations that the natural adrenal cortical hormones were.[28] The synthetic ketoles, however, correspond to the adrenal cortical hormones only with respect to their synergistic activity, but they are unable to substitute the adrenal cortical hormones in the survival test of adrenalectomized rats.

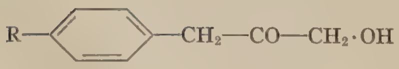
These results represent another example of the usefulness of the tissue culture technique for the evaluation of the effects of chemical variation of a single compound, as well as of a synergistic substance.

$CH_3—CO—CH_2\cdot OH$

II Acetole

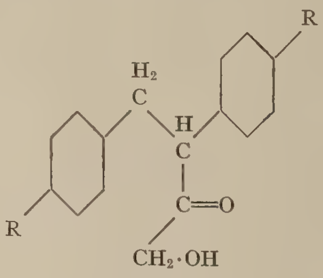


III Phenylketol



IV Phenylacetole

R = —H, —OH, —OCH$_3$



V Stilbanketol

The local anesthetics novocaine and xylocaine are another example of the possibility of differentiating two closely related compounds by this method. I have been asked whether it might be possible to demonstrate differences in their behavior as toxic agents for cells in a tissue culture. Because both compounds are organic amines,[29] they cause a vacuolization of the cell plasma in comparable dosages, as has been shown by Doctor Renate Lettré in a motion picture. In combination with a threshold dosage of colchicine, however, xylocaine exhibits a very pronounced synergistic effect, while novocaine is less active as a synergist[30] (*cf.* CHART 2). From this difference it is concluded that xylocaine—besides being a local anesthetic—is also active on the cell in some other way.

The first antagonistic effects we observed were caused by the addition of a second compound to two other types of mitotic poisons: the acriflavine and organometallic compounds. The antimitotic activity of acriflavine could be counteracted by adding nucleic acids (RNA & DNA),[31] while organometallic compounds, *e.g.* CH_3-Hg-Cl, lose their antimitotic or cell-damaging effects on the addition of cysteine[32] and other compounds containing SH-groups. Likewise, 8-hydroxyquinoline counteracts the antimitotic activity of organometallic compounds by forming complex compounds.[32] The compound 8-hydroxy quinoline, however, has a growth-inhibiting effect by itself because of its ability to react with metals present in the medium. A mixture of both, therefore,

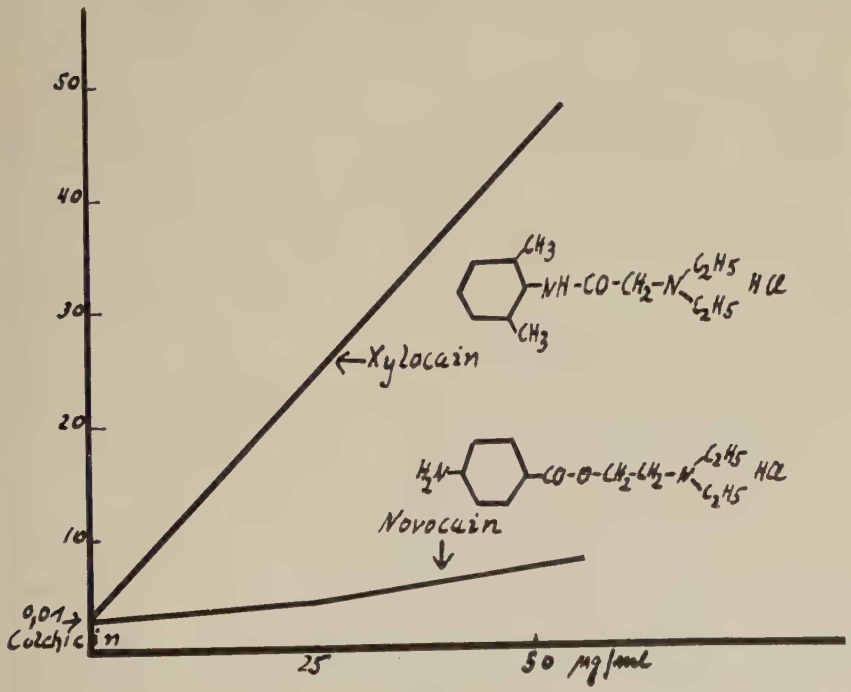


CHART 2

does not show any inhibitory effect (in concentrations inhibiting the growth completely if each compound is used alone). The antagonism between nucleic acids and acriflavine, as well as between cysteine and organometallic compounds introduces another subject for discussion: the rôle of the medium. Nucleic acids and compounds with free thiolgroups, which are antagonists of these types of mitotic poisons are present in the medium. From the quantitative point of view, the activity of the antagonist added depends on the amount of antagonists already present in the medium. If it were possible to use a medium which does not contain nucleic acids and compounds with free thiolgroups, but would still have its ability to stimulate the growth of the cultures, one would need a higher dosage of the respective antagonists in order to counteract the activity of the mitotic poison. It is conceivable that there are substances which act on cells only by their ability to block essential factors of the medium.

Several chemical factors are known to counteract the antimitotic activity of colchicine. Törö[33] prepared the so-called "corhormone," an extract from the heart of hog embryo, the "active" compounds of which should also be present in the medium (through embryonic extract). Although we have carried out numerous experiments in order to confirm the antagonistic effect of corhormone toward colchicine as described by Törö, we were unable to obtain this effect consistently. It might well be the case that some other as-yet-unknown factor of the medium is required to make this effect reproducible. This question seems to be rather complicated and requires further investigation. Another antagonist of colchicine is the sulfa drug Prontosil, which counteracts the induction of polyploidy by colchicine on plants (Bauch[34]). Using fibroblasts, we were not able to notice any antagonistic effect of Prontosil on colchicine-treated cultures.

In further studies on the elucidation of the analogy between muscle and spindle contraction, Landschütz, in our laboratory,[35] investigated the antagonistic action of cardiac poisons. Cardiac poisons stimulate the ATPase of the heart muscle.[36] At first[f] I thought that colchicine might inhibit this reaction, an assumption which could not be confirmed.[13, 37] Digitan and Digilanid did show antagonistic effects in concentrations which are almost damaging to the cells. In later experiments with strophantin and colchicine, however, we could not confirm this assumption.[38]

Although creatinphosphate is not an antagonist of colchicine, its addition to the medium—in the presence of colchicine—improves the shape of the spindle.[14] The addition of phosphagen has a growth-stimulating effect on the cultures, as judged by the increase of the number of mitoses.

The effects of ATP (sodium salt) on cells are difficult to interpret because of its fairly rapid degradation to ADP, AMP, inosinic acid, and NH_3, not to mention further transformation of the latter nucleotide to hypoxanthine. ATP itself is active for a few hours only; in concentrations of 3 mgm./ml. it causes a rigidity of the cell and stops movement within it, as shown in the motion-picture of Doctor Renate Lettré, while a dosage of 1 mgm./ml. does not produce this phenomenon. The addition of a mixture of colchicine (0.04 μg./ml.) and ATP (1 mgm./ml.) to explants of fibroblasts postpones the arrestment of the cells in metaphase for three to four hours as compared to fibroblasts sub-

TABLE 8

PERCENTAGE OF MITOSES AFTER THE ADDITION OF COLCHICINE ALONE (0.04 µg./ml.) AND THE MIXTURE OF COLCHICINE (0.04 µg./ml.) + ATP-Na (1 mg./ml.) TO FIBROBLASTS

Hours after addition	Colchicine	Colchicine + ATP
1	2.0	2.0
2	7.7	3.0
3	11.2	3.3
4	13.0	5.0
5	16.4	8.3
9	27.4	9.4
14	38.4	23.2

jected to the concentration of colchicine alone (*cf.* TABLE 8).[13] As far as we know this instance represents the only reproducible antagonism toward colchicine thus far observed.

In 1951 Murray, de Lam, and Chargaff[39] reported the antagonistic effect of meso-inositol toward colchicine on rat fibroblasts beginning 18 hours after addition of the compound. Under our experimental conditions we were unable, 24 hours after the addition of the meso-inositol, to confirm this finding on chicken fibroblasts. In 1953 Benitez, Murray, and Chargaff[40] described an antagonism between tropolone and colchicine. In 1950 and 1951 we investigated tropolone and some of its derivatives alone, and also in combination with

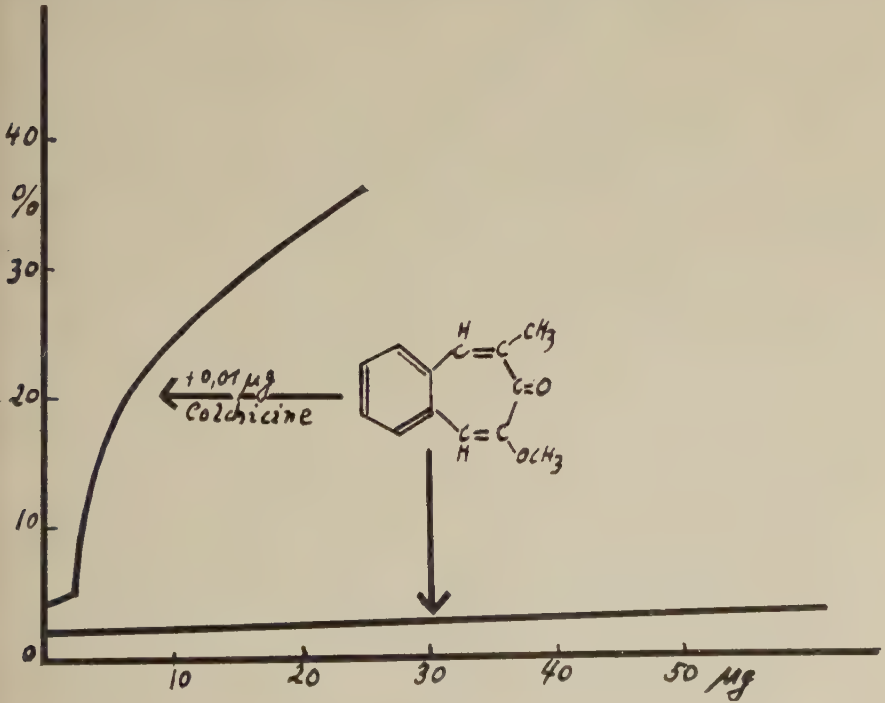


CHART 3

TABLE 9

DOSAGE IN μg./ml.

Pyocyanine	Colchicine	Percentage of mitoses after 24 hours
—	—	1.9 (0.7–4.0)
18	—	0.0
9	—	0.0
3	—	2.5
—	0.022	35.4
18	0.022	4.0
9	0.022	7.5
4.5	0.022	25.8
3	0.022	33.2

colchicine.[41] In contrast with the results of these authors we did not find any antagonistic effect produced by the derivatives; in most cases we found a synergism (*cf.* CHART 3, benzomethyltropolonemethylether). Tropolone itself is not a synergist, but on the other hand its antagonistic activity cannot be demonstrated beyond doubt, at least on chicken fibroblasts. The difference in the results obtained by the Chargaff group and by ourselves might raise the question of a difference in susceptibility between these compounds toward the fibroblasts of a rat and those of a chicken. The difference in the techniques used might also account—at least in part—for the divergence in the results.

The addition of pyocyanin to fibroblast cultures decreases the mitotic index.[42] In combination with colchicine there is a decrease of the number of arrested mitoses (*cf.* TABLE 9). Although this effect seems to be antagonistic, it is caused by the diminished mitotic activity of the culture, so that colchicine looses the substrate on which it can act.

Despite our systematic efforts we have failed to obtain an antimitotic effect by combining two compounds each of which has no activity by itself. It is possible that such a combination does exist. If such a combination could be found it should be of great value in understanding the mechanism of mitotic poisons.

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MITOTIC EFFECTS OF CERTAIN AMINO ACID ANALOGS IN TISSUE CULTURE*

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In a screening of about 50 amino acid analogs for selective damage to mouse sarcoma cells *in vitro*, among the most interesting which Jacquez and Mottram (1953) found were certain analogs of phenylalanine. One of these analogs, β-(2-thienyl)-*DL*-alanine (FIGURE 1), evokes a profound cytological response in sarcoma T241 in the C57 black mouse when it was administered in daily subcutaneous injections of 4 gm./kg. for five days (Jacquez, Stock, and Barclay, 1953). A massive polyploidization and multinucleation of the sarcoma cells occurred, individual mitoses having chromosome numbers in the hundreds and up to a score of poles. After treatment stopped, the giant cells rapidly decreased in frequency and were gone by the seventh day. This change occurred without pyknosis of the giant cells, suggesting that they disappeared by dividing into smaller cells. It was proposed that the β-(2-thienyl)-*DL*-alanine had caused giant cells to form by inhibiting cytokinesis more than by chromosomal reduplication and karyokinesis. It was necessary that the drug be administered daily in order for highly polyploid cells to develop. Although we found that the giant cells would grow in tissue culture, at least for a while, attempts to induce high polyploidization in tissue culture from the beginning by means of β-(2-thienyl-)-*DL*-alanine were unsuccessful. Even efforts to duplicate *in vitro* the possible sequence of changes in the extracellular concentration of β-(2-thienyl)-*DL*-alanine in the mouse (by exposing the cells to medium containing the agent for six hours and then to medium not containing the agent for 18 hours over a number of days) failed to produce the giant cells (Jacquez, unpublished).

However, our attempts to duplicate in tissue culture the cytological picture produced in sarcoma T241 in the mouse by another phenylalanine analog have been more successful. This analog is β-(*p*-fluorophenyl)-*DL*-alanine. Its structure is given in FIGURE 2, along with the formulas of the other two monofluorinated phenylalanines.‡ Among halogen-substituted phenylalanines, only the fluorophenylalanines could be competitively blocked in toxicity by *L*-phenylalanine (Jacquez and Mottram, 1953). The most toxic of the three isomeric monofluorophenylalanines was β-(*p*-fluorophenyl)-*DL*-alanine. T241 sarcomas in mice subjected to repeated injections of this agent have now been found to show, rather than the high polyploidy resulting from thienylalanine

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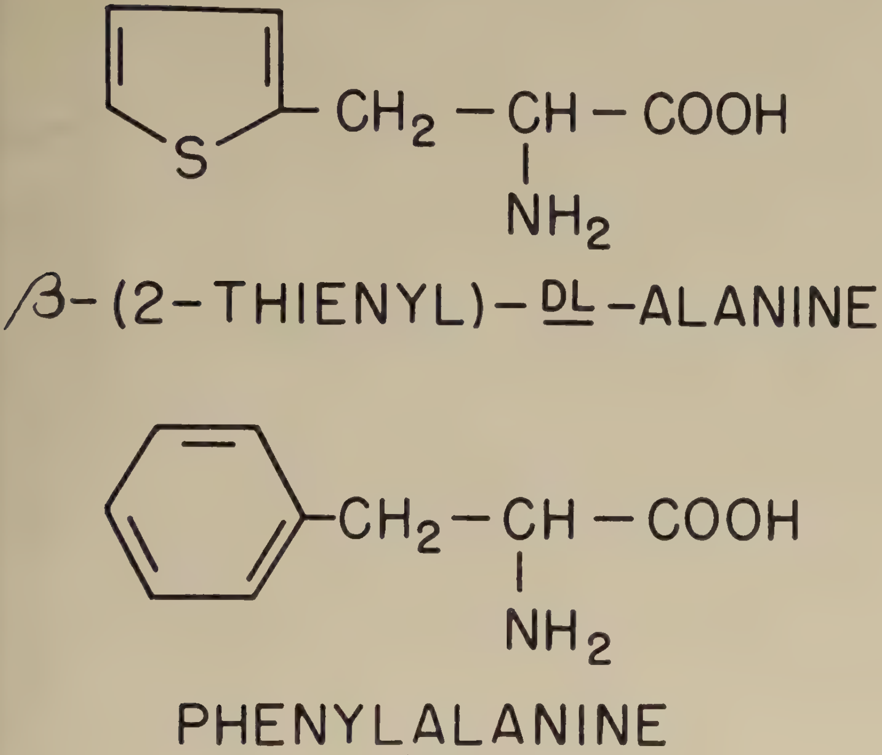


FIGURE 1

treatment, a possibly related phenomenon of metaphase arrest. Some of the arrested metaphases, in reverting to the resting condition, have established nuclei of necessarily doubled chromosome number, but extreme polyploidy did not develop. The mitotic effects in tissue cultures treated with the three fluorophenylalanines are the subject of this paper.

Experimental

Treatments of T241 sarcomas in C57 black mice were started six days after tumor implantation in the right groin. Injections of β-(*p*-fluorophenyl-*DL*-alanine suspended in a gum arabic solution were given subcutaneously in the left axilla or above the left scapula. The volume of dose was 0.2 ml. per 20 gm. mouse. Control mice were injected with the vehicle alone. The control mice were all sacrificed 10 days after tumor implantation. Of the four treated mice in TABLE 1, the first was sacrificed in a moribund condition on the ninth day after implantation, having received injections of 1.5 gm./kg. β-(*p*-fluorophenyl)-*DL*-alanine on each of two days and 1.0 gm./kg. for one day. The other three treated mice received one additional injection of 1.0 gm./kg. and were sacrificed in very poor condition on the tenth day after implantation. The tumors were fixed in alcohol-acetic acid 3:1, sectioned at 10 μ, and Feulgen-stained. Counts of about 1000 mitotic figures in each tumor gave the results of TABLE 1 in terms of mitotic phases.

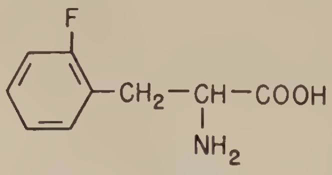
About 60 per cent of the mitotic figures in the treated tumors were metaphases, against 20 per cent in the controls. Some of the mitoses in the treated tumors were not permanently blocked in metaphase, because some anaphases and telophases were seen. Many blocked metaphases evidently reverted to the resting condition without anaphasic separation of chromosomes.

In a second experiment, the metaphase block was not so well-developed. Tumors treated with β-(*p*-fluorophenyl)-*DL*-alanine contained metaphases and prophases in nearly equal proportions, *i.e.*, in an intermediate condition between the controls and the treated tumors of the first experiment.

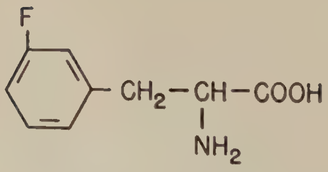
In tissue culture studies with the fluorophenylalanines, we exposed day-old cultures on coverslip inserts in roller tubes to the agents for one day and prepared permanent Feulgen-stained mounts, following for cultures of sarcoma-180 and embryonic skin the procedure given previously in this conference (Biesele, 1954). The culture technique was slightly different with sarcoma T241 and newborn mouse heart (Jacquez and Mottram, 1953).

Counts were made in the outgrowths to determine incidences of mitosis and of pyknosis, the distribution of mitotic phases, and the frequency of aberrant mitoses.

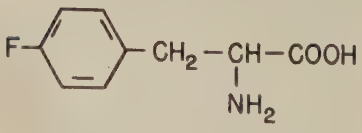
In cultures planted from newborn C57 black mouse heart, evidence was obtained for metaphasic delay under treatment with β-(*p*-fluorophenyl)-*DL*-alanine. TABLE 2 gives counts made in individual heart cultures treated with



β(o-FLUOROPHENYL)-DL-ALANINE



β(m-FLUOROPHENYL)-DL-ALANINE



β(p-FLUOROPHENYL)-DL-ALANINE

FIGURE 2

the agent at 0.25 mM, as well as a summary of the mitotic phase data in percentages.

Additional data concerning the metaphase delay in newborn heart and sarcoma T241 cultures are given in TABLE 3. Accumulation of metaphases

TABLE 1

MITOTIC PHASE DISTRIBUTION (1000 MITOSES) IN T241 SARCOMAS UNDER TREATMENT WITH β-(*p*-FLUOROPHENYL)-*DL*-ALANINE

	Prophase	Metaphase	Ana-telophase	Reconstruction	Reconstruction from blocked metaphase
Controls:					
	422	244	104	227	0
	425	193	75	307	0
	406	193	90	312	0
Treated:					
	196	589	4	18	193
	159	618	43	49	131
	285	582	13	12	108
	298	353	85	168	96

TABLE 2

MITOTIC PHASE DISTRIBUTION IN CULTURES OF C57 BLACK NEWBORN MOUSE HEART TREATED WITH β-(*p*-FLUOROPHENYL)-*DL*-ALANINE, 0.25 mM.

	Prophase	Metaphase	Anaphase	Telophase	Reconstruction
Counts:	11	31	4	3	50
	11	28	3	1	39
	13	45	2	0	49
	15	32	1	0	36
Percentage distribution:	13	36	3	1	47
Control percentages (untreated):	28	7	3	8	53

TABLE 3

MITOTIC PHASE DISTRIBUTION IN CULTURES OF C57 BLACK NEWBORN MOUSE HEART AND SARCOMA T241 TREATED WITH β-(*p*-FLUOROPHENYL)-*DL*-ALANINE AND *L*-PHENYLALANINE

β-(*p*-Fluorophenyl)-*DL*-Alanine	*L*-Phenylalanine	Cultures	Mitoses	Phases in per cent				
				Pro.	Meta.	Ana.	Telo.	Recon.
Heart:								
0 mM	0 mM	4	393	28	7	3	8	53
0.25	0	4	374	13	36	3	1	47
0.5	0	7	135	10	62	1	1	26
0.25	0.25	4	171	22	11	2	4	61
0.5	0.5	2	110	31	12	1	6	50
T241:								
0 mM	0 mM	4	351	27	21	1	2	48
0.25	0	10	278	29	43	1	4	23
0.25	0.25	2	273	21	28	0	9	42
0.5	0.5	3	293	20	24	5	12	39

was even more pronounced in heart cultures treated with 0.5 mM β-(*p*-fluorophenyl)-*DL*-alanine. It was also evident in T241 cultures treated with the agent at 0.25 mM. In both heart and T241 cultures treated with the agent, simultaneous administration of equimolar *L*-phenylalanine sufficed to return the mitotic phase distribution to about the control values.

In somewhat higher concentrations of β-(*p*-fluorophenyl)-*DL*-alanine, mitosis was suppressed in both heart and T241 cultures.

TABLE 4

MITOTIC SUPPRESSION BY β-(*p*-FLUOROPHENYL)-*DL*-ALANINE COUNTERACTED BY *L*-PHENYLALANINE IN CULTURES OF SARCOMA T241 AND C57 BLACK NEWBORN MOUSE HEART

β-(*p*-Fluorophenyl)-*DL*-Alanine	*L*-Phenylalanine	Mitotic		Pyknotic	
		(Nuclei per thousand)			
		T241	Heart	T241	Heart
Controls: 0	0	35	49	31	2
1.0 mM	0 mM	0	0	895	226
1.0	0.1	7	1	188	22
1.0	1.0	10	17	79	11
0.5	0	0	1	486	45
0.5	0.05	4	6	154	10
0.5	0.5	28	29	25	5
0.25	0	2	6	246	25
0.25	0.025	8	21	250	5
0.25	0.25	30	27	52	8

TABLE 5

COUNTS OF MITOSIS AND NUCLEAR PYKNOSIS IN CULTURES OF SARCOMA-180 AND EMBRYONIC MOUSE SKIN TREATED WITH FLUOROPHENYLALANINES

	Mitotic		Pyknotic	
	(Nuclei per Thousand)			
	S180	Skin	S180	Skin
Controls:	108	64	17	5
β-(*o*-Fluorophenyl)-*DL*-Alanine				
2.0 mM	0	0	360	370
1.0	23	22	130	41
0.5	28	34	89	37
0.25	66	43	24	18
β-(*m*-Fluorophenyl)-*DL*-Alanine				
2.0 mM	19	14	115	148
1.0	102	66	39	26
0.5	72	76	20	4
0.25	109	47	20	11
β-(*p*-Fluorophenyl)-*DL*-Alanine				
2.0 mM	0	0	793	678
1.0	0	0	434	398
0.5	30	9	55	94
0.25	47	15	41	22

TABLE 6

EFFECT OF β-(*m*-FLUOROPHENYL)-*DL*-ALANINE AND ATP ON MITOTIC PHASE DISTRIBUTION IN SARCOMA-180 CULTURES

β-(*m*-Fluorophenyl-*DL*-Alanine	ATP	Cultures	Mitoses	Phases in per cent				
				Pro.	Meta.	Ana.	Telo.	Recon.
0 mM	0 mM	2	243	21	10	0	5	64
1.0	0	3	389	30	26	2	4	39
1.0	1.0	4	600	34	37	2	3	26
0	0	2	192	31	12	1	4	53
0	1.0	2	182	37	14	1	6	42
2.0	1.0	3	276	22	60	1	1	17

This effect was counteracted by the addition of *L*-phenylalanine. Nuclear pyknosis was also diminished. TABLE 4 gives the results of nuclear counts in an antagonism experiment, each entry being the average of two cultures. It is seen that *L*-phenylalanine was approximately of equal effectiveness for heart and T241 cells in blocking the toxicity of β-(*p*-fluorophenyl)-*DL*-alanine.

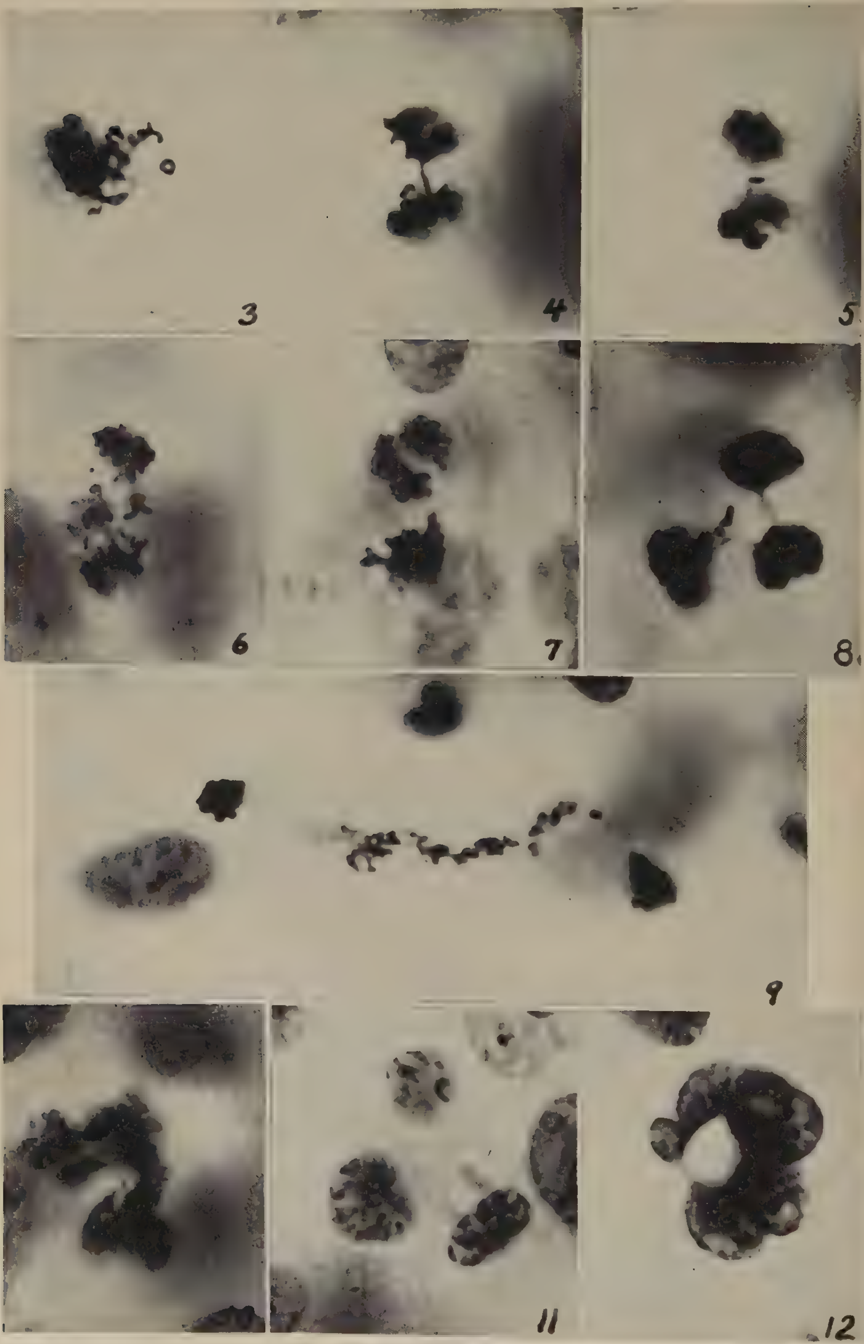
When the ortho-, meta-, and para substituted fluorophenyl-alanines were compared with respect to mitotic inhibition and the induction of nuclear pyknosis in cultures of T241 and heart, the para substituted compound was found to be about twice as effective as the other two. All three were slightly more toxic to the sarcoma cells than to the heart cells. Neither the ortho- nor the meta-substituted fluorophenylalanine appeared to cause a metaphase delay such as was seen with the para compound, although this point was not investigated in detail.

When the three agents were put on cultures of mouse sarcoma-180 and embryonic Ak mouse skin, results were somewhat different, as TABLE 5 shows. The most toxic was again β-(*p*-fluorophenyl)-*DL*-alanine, while the meta compound was least inhibitory to mitosis, and the ortho substituted compound had an intermediate effect. There was essentially no differential mitotic inhibition of sarcoma-180 with any one of the three agents, nor was there any marked metaphase delay.

The normal metabolite *L*-phenylalanine in equimolar concentration counteracted the mitotic inhibition and nuclear pyknosis induced by all three fluorophenylalanines. Protection was only partial against 2.0 mM β-(*m*-fluorophenyl)-*DL*-alanine. The antagonism was displayed about equally well with both sarcoma-180 and embryonic skin.

Adenosine triphosphate added in a concentration of 1.0 mM did not in general have this protective effect, although our results with the skin cultures were somewhat erratic. A surprising result was obtained in sarcoma-180 cultures treated with ATP in addition to β-(*m*-fluorophenyl)-*DL*-alanine. An increase in the relative frequency of metaphase figures occurred, capping a slight increase in incidence of mitosis, more marked at 2.0 mM β-(*m*-fluorophenyl)-*DL*-alanine than at 1.0 mM. The data are given in TABLE 6.

It should be noted that even without the ATP there was evident a tendency toward metaphase delay in sarcoma cultures treated with this meta substi-



FIGURES 3 to 12.

tuted analog of phenylalanine. Some of the delayed metaphases gave rise to restitution figures without anaphasic separation into groups of sister chromosomes. Some of these, derived from so-called "hollow-spindle" metaphases, formed toroid resting nuclei. Such a nucleus is illustrated in FIGURE 12. Other aberrant mitoses did undergo a partial anaphasic separation, although with such heavy chromosomal bridging that complete separation was impossible. The greatly lobed and bridged reconstruction nucleus of FIGURE 10 illustrates this effect. Such aberrant postmetaphasic mitotic figures made up 61, or 34.8 per cent, of the 175 postmetaphasic figures in three sarcoma-180 cultures treated with 1.0 mM β-(*m*-fluorophenyl)-*DL*-alanine. There were 88, or 48.9 per cent, aberrant figures among the 180 postmetaphases in four sarcoma cultures treated with 1.0 mM ATP in addition to 1.0 mM β-(*m*-fluorophenyl)-*DL*-alanine. Aberrant figures made up 44, or 84.6 per cent, of 52 postmetaphases in three sarcoma cultures treated with 2.0 mM β-(*m*-fluorophenyl)-*DL*-alanine in addition to 1.0 mM ATP.

Because of the possibility that some metabolite derived from fluorophenylalanine was actually responsible for the metaphase block observed *in vivo* and perhaps for the mitotic effects in tissue culture, several experiments were carried out. It was known, for example, that inorganic fluoride appeared in the urine of rats after the administration of para-substituted fluorophenylalanine (Armstrong and Lewis, 1951). Of course, the cited antagonism between *L*-phenylalanine and the fluorophenylalanines for both the mitotic inhibition and the metaphase delay might tend to rule out inorganic fluorides. Fluorophenylalanine might also conceivably be degraded to fluoroacetate in the animal.

Cultures of sarcoma-180 treated with sodium fluoride for one day appeared undamaged at 0.1 mM, showed a nearly complete suppression of mitosis at 5.0 mM, but had only a slight reduction in incidence of mitotic nuclei at 1.0 mM. Mitotic aberrations were not seen in cultures treated with 0.1 mM NaF and were not excessive, although they did occur, in cultures treated with 1.0 mM NaF. Sodium fluoroacetate was nearly without effect on sarcoma mitoses through 5.0 mM, causing only a slight reduction in mitotic incidence but no increase in aberrations. Because a greater suppression of mitosis had been noted with the ortho- and the para-substituted fluorophenylalanines at 1.0 mM than with sodium fluoride and sodium fluoroacetate, the latter compounds do not appear to have been primarily responsible for the effects seen with fluorophenylalanine.

FIGURE 3. Prometaphase with one ring chromosome in sarcoma-180 culture treated with β-(*o*-fluorophenyl)-*DL*-alanine at 1.0 mM. 1350 ×.

FIGURE 4. Anaphase with bridge in sarcoma-180 culture grown with liver explant and treated with 0.5 mM β-(*p*-fluorophenyl)-*DL*-alanine. 1350 ×.

FIGURE 5. Different focal level of mitosis in FIGURE 4, showing one chromosomal fragment of four present. 1350 ×.

FIGURE 6. Anaphase with 16 fragments or lagging chromosomes, from same culture as FIGURE 4. 1350 ×.

FIGURE 7. Late telophase or early reconstruction figure, with three chromosome groups, as though derived from mitosis in FIGURE 6. Same culture as FIGURE 4. 1350 ×.

FIGURE 8. Tripolar late telophase, with two bridges and two fragments, in sarcoma-180 culture grown with liver explant and exposed to 0.5 mM β-(*p*-fluorophenyl)-*DL*-alanine. 1350 ×.

FIGURE 9. Reconstruction figure, with long chain of chromosomal material between the two separating sister groups. From same sarcoma-180 culture as FIGURE 8. 1350 ×.

FIGURE 10. Heavily bridged reconstruction figure in sarcoma-180 culture exposed to 1.0 mM β-(*m*-fluorophenyl)-*DL*-alanine plus 1.0 mM ATP. 1350 ×.

FIGURE 11. Tripolar reconstruction figure in sarcoma-180 culture grown with liver explant and treated with 1.0 mM β-(*m*-fluorophenyl)-*DL*-alanine. 1350 ×.

FIGURE 12. Toroid resting nucleus in sarcoma-180 culture treated with 2.0 mM (β-(*m*-fluorophenyl)-*DL*-alanine. 1350 ×.

TABLE 7

NUCLEAR COUNTS IN SARCOMA-180 CULTURES, WITH AND WITHOUT ACCOMPANYING LIVER CULTURES, EXPOSED TO FLUOROPHENYLALANINES. EACH ENTRY IS THE MEAN OF TWO CULTURES

	Mitotic		Pyknotic	
	(Nuclei per Thousand)			
	S180	S180 and liver	S180	S180 and liver
Controls:	93	87	19	46
β-(*o*-Fluorophenyl)-*DL*-alanine				
1.0 mM	10	14	268	185
β-(*m*-Fluorophenyl)-*DL*-alanine				
2.0 mM	14	22	148	165
1.0	25	75	107	93
β-(*p*-Fluorophenyl)-*DL*-alanine				
1.0 mM	0	0	409	420
0.75	3	17	230	182
0.5	18	24	104	129
0.25	49	37	38	30

Another approach was tried on the theory that the liver might convert fluorophenylalanine into a substance responsible for the *in vivo* cytological picture. Accordingly, two explants of newborn mouse liver were planted on either side of a sarcoma-180 explant on coverslip inserts in roller tubes. Other tubes held cultures of sarcoma-180 alone. When sets of these cultures were dosed with the fluorophenylalanines, nearly identical results were obtained in the sarcoma cultures with and without neighboring liver explants with respect to mitotic inhibition and the induction of pyknosis (TABLE 7).

The relative proportions of mitotic phases in the sarcoma cultures were also largely uninfluenced by presence of the liver explants, except for those cultures treated with β-(*p*-fluorophenyl)-*DL*-alanine. In these cultures, metaphase delay occurred (TABLE 8). In the presence of liver, sarcoma-180 cultures responded to β-(*p*-fluorophenyl)-*DL*-alanine as T241 or heart cultures by themselves responded.

Another interesting effect was noted in sarcoma cultures treated with the fluorophenylalanines in the presence of liver cultures. This was the occurrence of tripolar mitoses. There were 14 tripolar postmetaphase figures noted, along

TABLE 8

DISTRIBUTION OF MITOTIC PHASES IN CULTURES OF SARCOMA-180, WITH AND WITHOUT ACCOMPANYING LIVER CULTURES, TREATED WITH β-(*p*-FLUOROPHENYL)-*DL*-ALANINE

β-(*p*-Fluorophenyl)-*DL*-alanine	Liver	Cultures	Mitoses	Phases in per cent				
				Pro.	Meta.	Ana.	Telo.	Recon.
0 mM	Absent	4	362	31	7	1	4	57
0	Present	4	348	33	10	2	4	51
0.5	Absent	6	269	59	12	2	5	22
0.5	Present	5	427	29	38	4	4	25

with 546 bipolar figures, in 14 of 20 sarcoma cultures grown with liver and treated with the fluorophenylalanines. No tripolar mitosis was seen among 223 post metaphases in four control cultures of sarcoma-180, among 198 postmetaphases in four sarcoma cultures grown with liver but not treated otherwise, nor among 255 postmetaphase figures in 17 sarcoma cultures treated with fluorophenylalanines in the absence of liver cells. The effect was noted with all three fluorophenylalanines. In this respect, sarcoma-180 in the presence of liver and fluorophenylalanine again approaches the T241 sarcoma cytologically. In control cultures of the latter tumor, tripolar mitoses make up about one tenth of all mitotic figures.

Tripolar mitoses in sarcoma-180 cultures under this combination of liver cells and fluorophenylalanine are illustrated by FIGURE 8, in which the three telophasic chromosome groups are connected by two bridges and there are two lagging chormosomes or fragments, and by FIGURE 11, in which there are three sister nuclei in reconstruction.

Other mitotic aberrations in sarcoma-180 cultures treated with fluorophenylalanines may be noted. In FIGURE 3 there is a ring chromosome in a prometaphase figure; this culture had been treated with β-(*o*-fluorophenyl)-*DL*-alanine. FIGURES 4 and 5 are two focal levels of an anaphase in which one bridge and four chromosome fragments were noted. This mitosis, like those of FIGURES 6, 7, 8, and 9, occurred in a sarcoma culture treated with β-(*p*-fluorophenyl)-*DL*-alanine in the presence of liver cells. The anaphase of FIGURE 6 contains 16 fragments or lagging chromosomes between the two sister groups. FIGURE 7 is of a later stage, a late telophase or early reconstruction figure in which the central nucleus of the three might conceivably have been formed from a group of lagging chromosomes such as those in FIGURE 6. Another reconstruction or late telophase figure with a long string of chromosomal material between the two small terminal groups is seen in FIGURE 9.

Discussion

From the effects on mitosis produced by the fluorophenylalanines, and from the blocking of these effects by the normal amino acid, *L*-phenylalanine, it may be tentatively concluded that phenylalanine or a metabolic derivative of it plays an important role in mitosis in mouse cells. A similar conclusion has been reached by Kieler (1953a, b) for other amino acids.

The delay in metaphase seen with the fluorophenylalanines under certain conditions suggests that spindle behavior or the centromere-spindle relationship was affected. The enhancement by ATP of the metaphase delay induced by β-(*m*-fluorophenyl)-*DL*-alanine is reminiscent of that produced in sarcoma-180 cultures by the combination of adenosine and 2-aminoadenosine (Biesele, Berger, Clarke, and Weiss, 1952). Indeed, the cytological details, including the formation of toroid resting nuclei by reversion of arrested hollow metaphases, were closely similar. In view of the protection afforded by ATP against certain spindle poisons (*cf.* Lettré, 1952), it is surprising that ATP acted otherwise with this fluorophenylalanine. Perhaps the ATP provided the wherewithal for production of a more active agent from the β-(*m*-fluorophenyl)-*DL*-alanine. A metabolic conversion of β-(*p*-fluorophenyl)-*DL*-alanine by liver cells is suggested by results of that combination on sarcoma-180, but proof of the hypothesis must await isolation and test of the hypothetical derivative. If the substance in question had been fluoroacetate or inorganic fluoride, mitotic effects should have been produced by one of them at concentrations below those at which the fluorophenylalanines were effective.

The problem of chromosome breakage by these chemicals, as illustrated in FIGURES 3, 4, 5, 6, and 8 is a complex one. The frequency of bridging and fragmentation in treated sarcoma-180 cultures is higher than that in control cultures, but it is not extreme. Are these fluorophenylalanines to be regarded as mutagenic, any more than aspartic acid, which Kieler (1953c) found to cause chromosome breakage after an initial stimulation of mitosis in chick embryo fibroblasts cultured in dialyzed medium? Of course, chromosome-breaking chemicals have been defined as "mutagenic" by Oehlkers (1949) and D'Amato (1950). But in this case we lean toward an interpretation based on Koller's study (1953) of repeatedly occurring dicentric chromosomes in a rat tumor. The aberrations always appeared in the tumor in the rat, but failed to occur in tissue culture. This "spontaneous" chromosome breakage in the tumor cell he attributes to metabolic disturbance. Such inherent cellular "instability" would be enhanced by the presence of appropriate antimetabolites such as the fluorophenylalanines in our examples. It is noteworthy in this respect that our embryonic skin cultures often did not show mitotic aberrations in parallel with the sarcoma cultures under treatment, despite a fair agreement in mitotic inhibition. Koller (1953) states that spindle abnormalities could also result from cellular instability, and we note that in our material metaphasic delay (spindle abnormality?) and later chromosomal bridging and fragmentation have tended to show up together in cultures treated with the fluorophenylalanines.

Summary

Metaphasic delay caused by β-(*p*-fluorophenyl)-*DL*-alanine administered to sarcoma T241 *in vivo* could be produced in cultures of T241 and heart by the same agent, but not in sarcoma-180 and embryonic mouse skin without other changes of the conditions of culture. In the presence of liver cells and β-(*p*-fluorophenyl)-*DL*-alanine, sarcoma-180 mitoses were slowed in metaphase. A similar metaphasic delay, sometimes resulting without division in toroid resting nuclei, was noted in cultures of sarcoma-180 treated with β-(*m*-fluorophenyl)-*DL*-alanine, especially when this agent was supplemented with ATP. Additional mitotic aberrations, including chromosome bridges and breaks, occurred in sarcoma-180 cultures under treatment with either the ortho-, meta-, or para-substituted fluorophenylalanines. Mitotic inhibition and metaphase arrest caused by the fluorophenylalanines could be blocked by *L*-phenylalanine. Of possible metabolic derivatives of fluorophenylalanine, fluoroacetate and inorganic fluoride did not appear to be responsible for the mitotic effects observed.

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STUDIES ON INHIBITION OF THE COLCHICINE EFFECT ON MITOSIS*

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The group of experiments here summarized represents a part of a collaborative program designed to investigate the mechanism of mitosis from the standpoints of cell physiology and of cell chemistry. A time-honored approach to this problem has been to interfere with the process of mitosis by diversified chemical agents designated (because of this action) as mitotic poisons: more recent investigations have included compounds which will inhibit or reverse the effects of the mitosis inhibitors.

With this general intent, the adult rat fibroblast (from subcutaneous areolar tissue) was selected as physiological material, and colchicine as a mitosis inhibitor whose action as a spindle poison, producing prolonged metaphase arrest, is reasonably clear-cut. No attempt can be made in this paper to review the vast literature on mitotic poisons, or the literature which has accumulated on colchicine alone since the papers of Dustin and of Lits, in 1934, focussed attention upon its effects on cell division. For the latter, the Eigsti bibliographies of colchicine (1947, 1949), *The Tissue Culture Bibliography* (1953), and a brief review by Levine (1951) may lead into the subject.

Method and preliminary observations. The conclusions reported below on the action of mitosis poisons and their inhibitors are all based on determinations of the mitotic coefficient (ratio of dividing to resting cells), and analyses of the dividing cell population in terms of phase percentages. Details of the culture and counting techniques used here are essentially the same as those described by Murray, de Lam, and Chargaff (1951). As stated in that communication, every experimental series includes a saline control; moreover, wherever two agents are used simultaneously, as for blocking purposes, they are also tested separately at the same time on the same biological material. In other words, a typical experiment consists of the following four groupings: (1) colchicine alone; (2) colchicine plus antagonist; (3) antagonist alone, (4) saline (TABLE 1, FIGURE 8).

For reliable interpretation of these experimental data obtained by the tissue-culture method it has been found necessary to establish rather broad-based numerical relationships between resting and dividing cells in control and colchicine-exposed tissue, and to apply the same quantitative treatment rigorously with each agent. Counts of the order of 5,000 cells for each grouping in an experimental series are necessary if statistically valid generalizations are to be made (TABLE 1). Smaller numbers give reliable data only for gross toxicity tests, but not for analyses of drug effects on mitosis. Qualitative observations considered alone can be quite misleading in such a system; this fact need not be surprising since, according to Lewis (1951), the time occupied by mitosis in a

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TABLE 1

COLCHICINE 2×10^{-6} AGAINST MUCO-INOSITOL 2×10^{-4} AND INOSITOL LIPIDE 2×10^{-4}

Agent	Total cells	Total mitoses	Mitotic coefficient
Colchicine 2×10^{-6}	10783	4023	0.373
Colchicine 2×10^{-6} + muco-inositol 2×10^{-4}	11108	3492	0.314
Colchicine 2×10^{-6} + inositol lipide 2×10^{-4}	10538	3881	0.368
Muco-inositol 2×10^{-4}	8680	128	0.014
Inositol lipide 2×10^{-4}	8715	177	0.020
Control saline	7799	240	0.030

normal fibroblast may vary from 94 to 195 minutes—a factor of more than two. Our own observations indicate that in addition to individual variation among donor animals, different cultures of the same age from the same strain of areolar fibroblasts may vary markedly in mitotic rate at a given time; and, over a period of a month's cultivation, descendents of the same cultures may fluctuate in mitotic rate by a factor of two or three. The mitotic coefficient is found also to fluctuate quite perceptibly over a period of 44 hours, when counts are made at 4-hour intervals (FIGURE 1).

All these tests are run on double cover-slip (Maximow-type) cultures which are transferred from stock roller-tubes five days before being exposed to test conditions. During this time they have received one feeding, and their growth has become stabilized after transfer. The experimental agents and the control saline are incorporated into the feeding solution as two parts in five: thus these cells are regularly maintained under physiological conditions in which a supply of normal metabolites somewhat comparable to that obtainable *in vivo* is available. Preliminary experiments are always performed to determine effective range and toxic level; definitive tests are carried out only at concentrations whose effects are reversible. These precautions are found necessary to make sure that only the specific action of the experimental agents shall be brought into play.

Colchicine. For colchicine* in this system, a final molarity of 2×10^{-6} is the most effective reversible concentration; this molarity has been adopted for standard use. Rate counts run on colchicine-treated cultures at 4-hour intervals over a period of 72 hours show that the peak of metaphase accumulation is reached at about 20 hours; this level remains steady for a short time and thereafter declines. Consequently a 20-hour observation period was adopted as covering the significant range for this compound and its inhibitors.

Other potential inhibitors. Tropolones. With norms established for colchicine and controls, several other agents chemically related to colchicine or reputed to be similar in effect were tested on this biological system. Three simple colchicine analogs,† tropolone, tropolone methyl-ether, and 4,5-tetramethylene tropolone (FIGURE 2) were investigated. At 10^{-3} M, tropolone is severely toxic; but, unlike colchicine, these simple cycloheptene compounds produce no

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* U.S.P., obtained from the Inland Alkaloid Company, Tipton, Ind.

† We are very grateful to Professor W. v. E. Doering of Yale University for supplying these compounds.

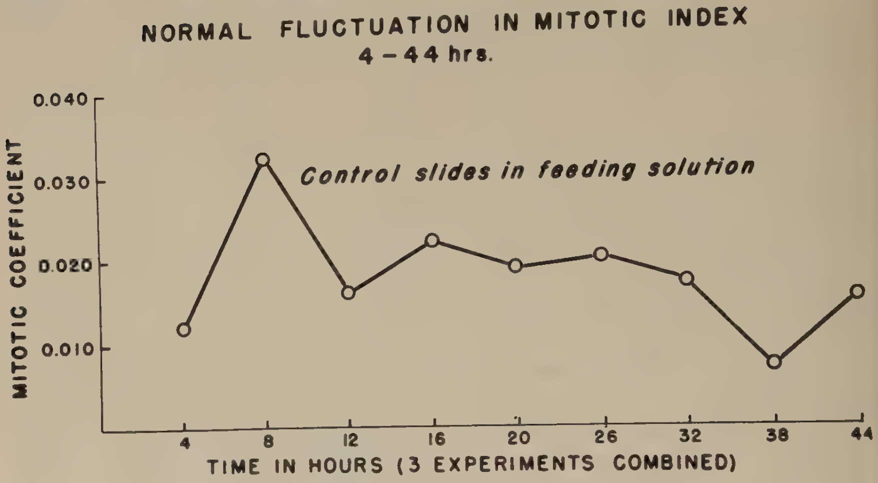
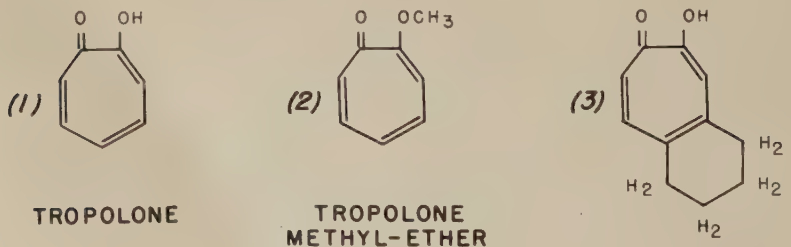


FIGURE 1

metaphase arrest. On the contrary, tropolone and 4,5-tetramethylene tropolone at their most effective reversible concentrations, which range from 10^{-4} to 10^{-5} M, may reduce the mitotic coefficient by 25 to 50 per cent as compared to the controls. At the same time, the phase distribution is altered, so that the percentage of metaphases is sharply reduced in favor of premetaphase and postmetaphase figures. In fixed tropolone-exposed cultures the spindle appears sharper and clearer than in controls. From these observations it might be reasoned that tropolone and tetramethylene tropolone accelerate mitosis, exerting an influence particularly on the spindle. Tropolone methyl-ether appears to be ineffective in all concentrations from 10^{-3} to 5×10^{-6} M (FIGURE 3, TABLE 2).

Gammexane. This hexachloro derivative of muco-inositol, gamma-hexachlorocyclohexane, was shown by Nybom and Knutsson (1947) to exert a colchicine like effect on mitosis in the onion root tip. (For the constellation of the chlorine atoms in gammexane, compare Van Vloten *et al.*, 1948.) We have tested gammexane similarly on rat fibroblasts and found it to be completely



(1) TROPOLONE

(2) TROPOLONE METHYL-ETHER

(3) 4,5 TETRAMETHYLENE TROPOLONE

FIGURE 2

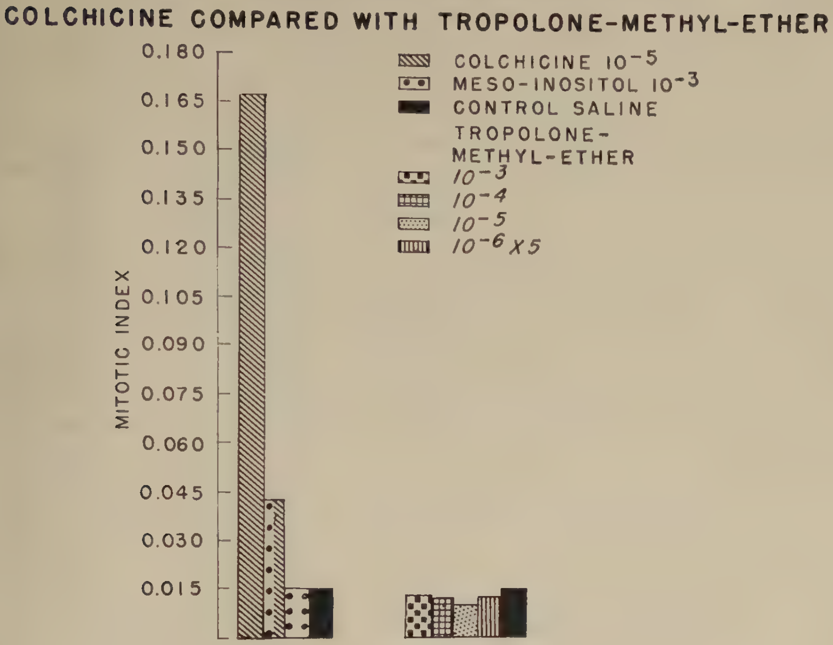


FIGURE 3

TABLE 2

TROPOLONE METHYLETHER 10^{-3}, 10^{-4}, 10^{-5}, 5×10^{-6}

Agent:	Total Cells	Total Mitoses	Mitotic Index
Tropolone Methylether 10^{-3}	6724	94	0.013
Tropolone Methylether 10^{-4}	6522	83	0.012
Tropolone Methylether 10^{-5}	6184	74	0.011
Tropolone Methylether 5×10^{-6}	6779	89	0.013
Control Saline	6512	101	0.015

ineffective at concentrations ranging from 10^{-6} M to 2×10^{-5} M. Because of its low solubility it was incorporated into the medium by means of cellosolve. Cellosolve by itself at the experimental concentrations has no effect on mitosis (see also Friedmann and Simon-Reuss, 1951). The delta and epsilon isomers of gammexane were tested similarly at a concentration of 2×10^{-6} M, and found to have no effects detectable by mitotic coefficient or phase percentage measurements.

*Protoanemonin.** This lactone, which has been reported as a growth inhibitor for bacteria (Holden, Seegal, and Baer, 1947), was tested for its effects on mitosis. In our system it was found to have, at high concentration, a transient action in raising the mitotic coefficient (FIGURE 4) above the normal, which

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* We are indebted to Doctors Margaret Holden and Beatrice Seegal, of the Department of Microbiology for our supply of this compound.

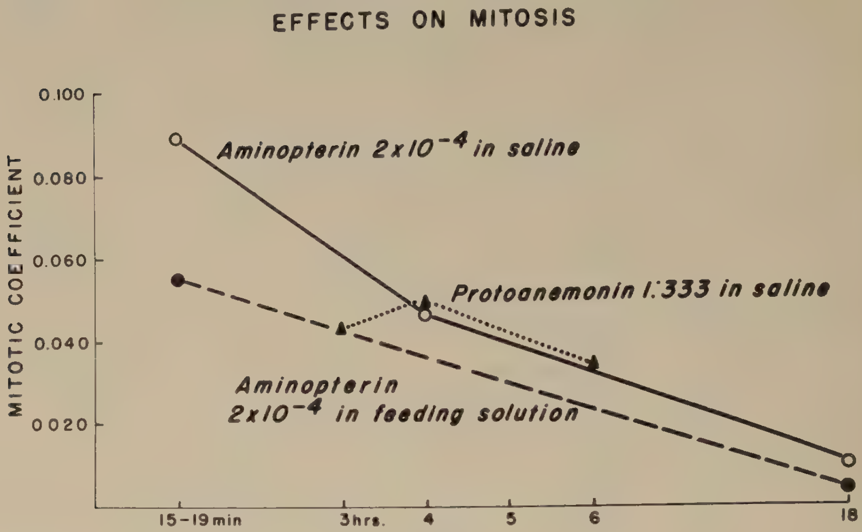

FIGURE 4

fluctuates around 0.020 (FIGURE 1); but it had no influence on phase percentages. These percentages retained essentially the same distribution as in the controls, with no indication of metaphase arrest. The presence of feeding solution in the medium has a cushioning effect on the drug action: marked effects of protoanemonin were noted only when it was added in saline alone. For this reason it seems likely that protoanemonin acts to retard the mitotic process rather than to reduce the duration of the interphase period. It has been reported by Erickson (1948) that treatment of Zea Mays seedlings with protoanemonin results in disappearance of mitochondria and premetaphase inhibition.

*Aminopterin.** Since this folic acid antagonist was described by Hughes (1950) as a complete spindle inhibitor in fibroblasts from embryonic chicks, it seemed desirable to test it on our system. Results here indicate that it has a transient colchicine-like effect (FIGURE 4), as Hughes reports. But at the most favorable concentration, 2×10^{-4} M, it is very much less effective than colchicine at 2×10^{-6} M. As with protoanemonin, the tests were run both with and without feeding solution in the medium. For these two relatively ineffective compounds the results are of the same order, but action is intensified in the absence of feeding solution. In the first 20 minutes of exposure to Aminopterin, a metaphase arrest occurs, but this effect disappears within the first four hours.

Colchicine Inhibitors

Since colchicine is one of the most uniformly effective and predictable of the mitotic poisons as well as the most spectacular in its action, it seemed that blocking attempts should be directed toward this end. In the rat fibroblast

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* Doctor James Ruegsegger, of Lederle Laboratories, kindly supplied the aminopterin.

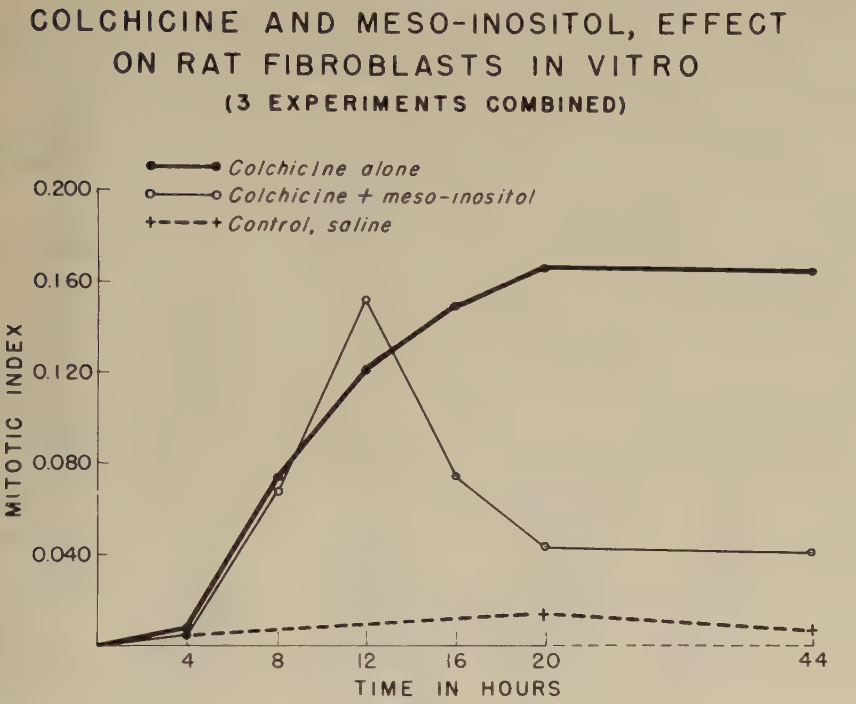


Figure 5

system, three compounds, widely different in chemical structure, have so far been shown to counteract the mitotic effects of colchicine when administered simultaneously with it.

Meso-inositol. Since Chargaff *et al.* (1948) had demonstrated in Allium cepa an inhibition of the colchicine effect by meso-inositol, a finding not generally accepted by later workers, this compound was the first to be tested in the rat fibroblast preparations. As reported by Murray, de Lam, and Chargaff (1951), it was found to be very effective here also as a blocking agent, reducing the mitotic coefficient of colchicine-treated cultures by 50 to 75 per cent in 20 hours. Time sequence experiments show (FIGURE 5) that with meso-inositol present in

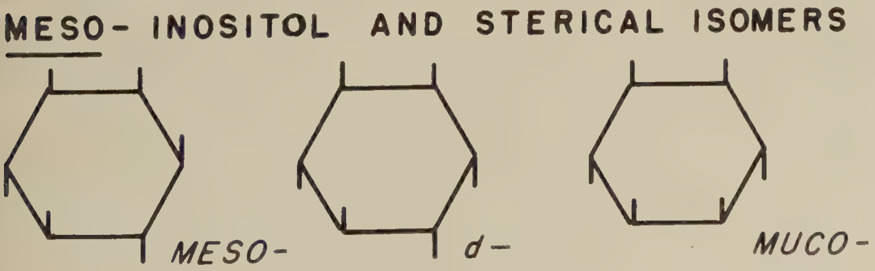
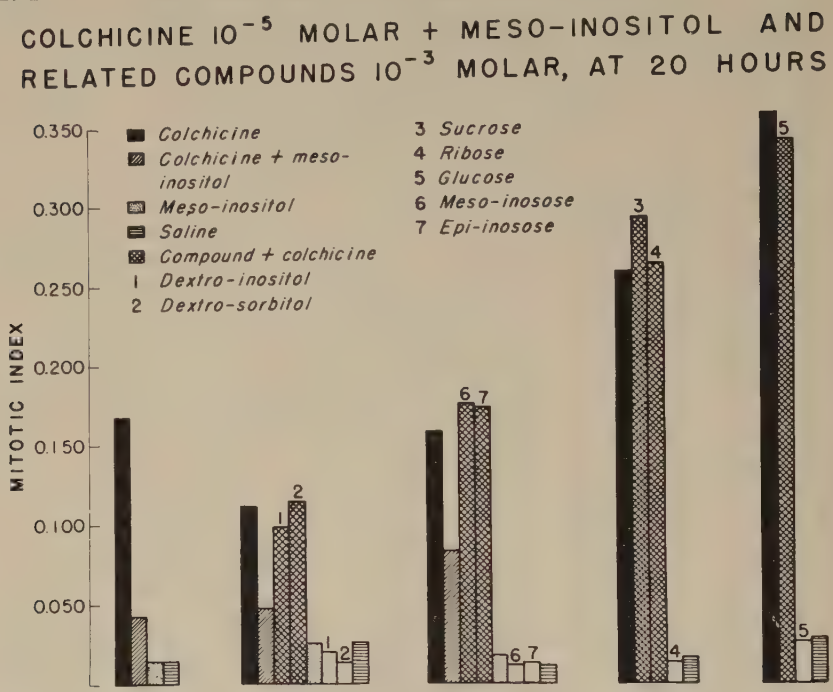


Figure 6



COLCHICINE 10^{-5} MOLAR + MESO-INOSITOL AND RELATED COMPOUNDS 10^{-3} MOLAR, AT 20 HOURS

FIGURE 7

the medium at 2 × 10^{-4} M and colchicine at 2 × 10^{-6} M, the mitotic coefficient rises parallel to that in colchicine alone up to 12 hours, then abruptly descends. This 12-hour period coincides with the average duration of the interphase period as reported by Fell and Hughes (1949) for mouse cells *in vitro;* consequently it was suggested that meso-inositol may replace some necessary metabolite inactivated by colchicine, and thus allow the cell to prepare once more for division.

In bringing about such repair, the sterical structure of meso-inositol appears to be of extraordinary importance, since two of its isomers, dextro- and muco-inositol (FIGURE 6) are essentially ineffective.* So also are the keto-derivatives epi-inosose and meso-inosose. Glucose, sucrose, ribose, and D-sorbitol produce no significant alteration of the colchicine-effect (FIGURE 7). In their 1951 communication the authors suggested that, in the course of its utilization by the cell, inositol might be converted to a derivative, perhaps a phosphoric-acid ester, and utilized in this form. Following this suggestion, a preparation of the inositol lipide of soybean (Hawthorne and Chargaff, 1954) was tested; it is apparent that this compound, when available exogenously, is completely ineffective to prevent or reverse the action of colchicine (TABLE 1).

Analysis of the meso-inositol data by phase percentages and by another numerical method which will be described in a later communication indicate

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* We are very grateful to Professor H. O. L. Fischer, of the University of California, Berkeley, Calif., for a specimen of muco-inositol.

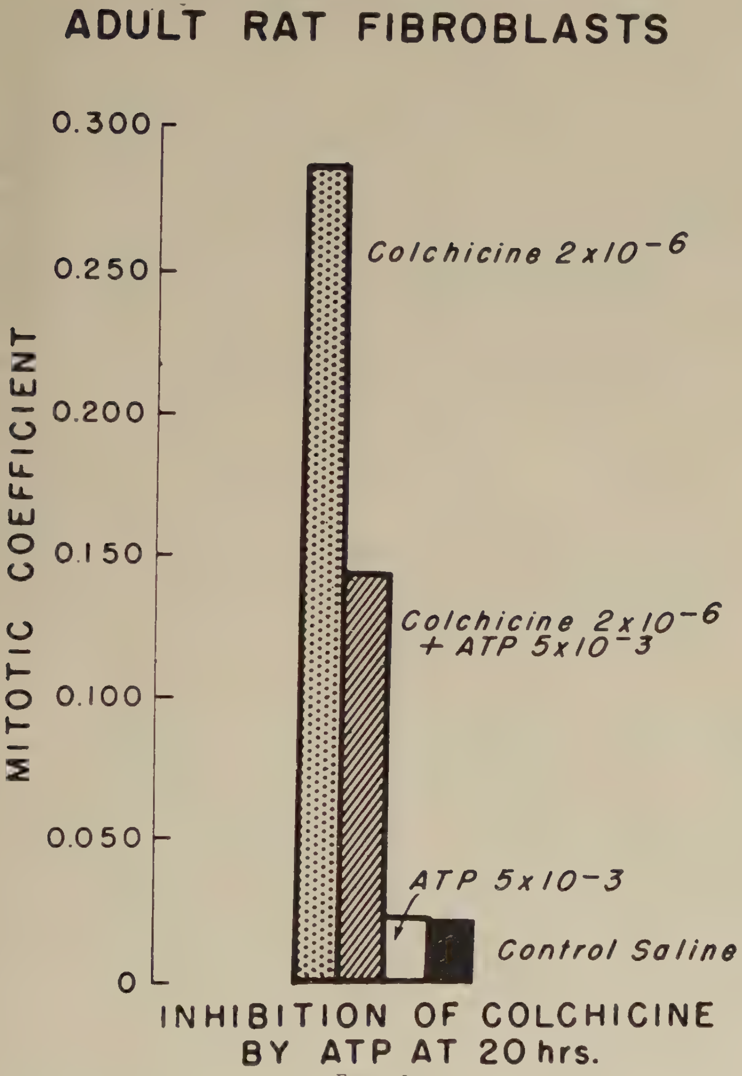

FIGURE 8

that meso-inositol tends to shorten the interphase period. However, its action in antagonizing colchicine is not intensified by increasing its concentration tenfold, or by administration for as much as two weeks in advance of the exposure to colchicine; apparently meso-inositol is not stored by fibroblasts.

Administered simultaneoulsy with protoanemonin, meso-inositol does not alter the mitotic coefficient. But when administered with Aminopterin during this compound's early period of transient metaphase arrest, it reduces the mitotic coefficient by 60 per cent—a figure quite comparable to its reversal effect on colchicine. A short metaphase arrest induced by Aminopterin is reported also by Jacobson (1952), who was able to block it completely with the Leuconostoc citrovorum factor.

As might be expected, gammexane and its delta and epsilon isomers, inactive alone, were unaffected by the addition of meso-inositol.

Adenosine triphosphate (*ATP*). Since Brachet's suggestion, in 1944, of a possible role of ATP (similar to the actomyosin relationship) in the contractile processes involved in cell division, the subject has figured frequently in discussion. Lettré, who is of the opinion that "spindle contraction, leading to the separation of the chromosomes is . . . a process similar to muscle contraction" (1952) has reported in a note (Lettré and Albrecht, 1951) that the presence of ATP with colchicine, in embryo chick fibroblast cultures, reduces the percentage of mitoses drastically from the figure in colchicine alone. However, Weber (1953) in motion pictures of glycerol-killed fibroblast models, has produced

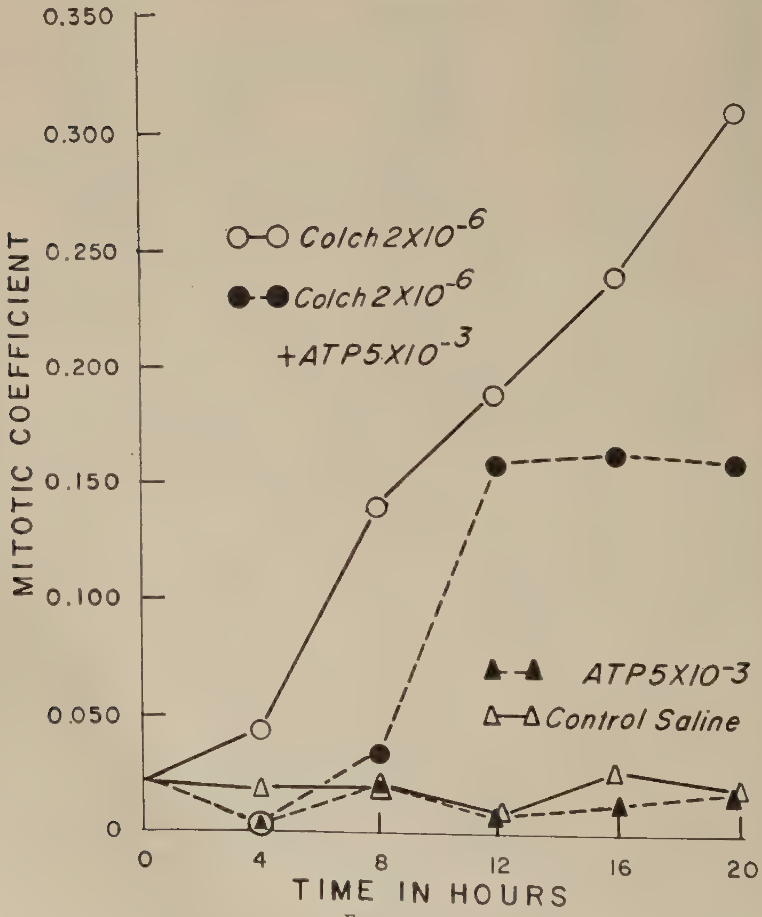

FIGURE 9

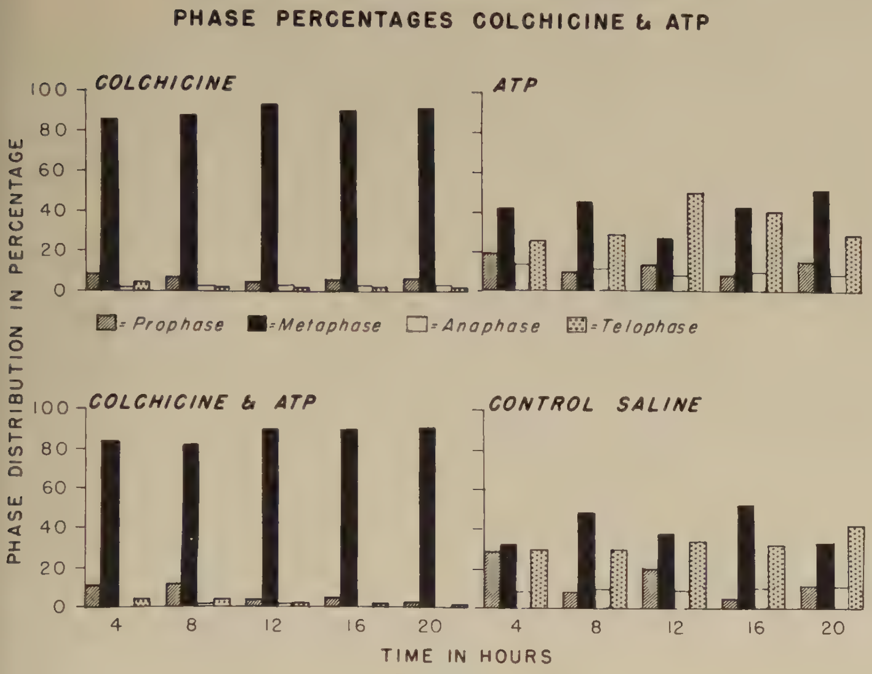

FIGURE 10

striking evidence that ATP (at *ca.* 10^{-2} M) induces a general contraction of the cytoplasm of the resting cell.

Though in tissues and tissue fluids ATP and ATPase are omnipresent in physiological quantities, it seemed desirable to test the agent in our system, using the high concentrations that have been reported as effective to induce contraction in nonmuscular tissues. In the customary 20-hour testing runs it was found that ATP at 10^{-2} M was rapidly and severely toxic. Many cellular abnormalities were produced, especially condensed pyknotic resting nuclei eccentrically located, and irregular but deeply staining ectoplasmic cell borders—a picture that was interpreted to indicate extreme cytoplasmic contraction. At 5×10^{-3} M, ATP alone is not toxic; in fact, no effect at all is demonstrable at the end of 20 hours. The group to which both colchicine and ATP are added shows approximately a 50 per cent reduction in mitotic coefficient at this time, which marks the peak of the metaphase arrest by colchicine (FIGURE 8), but phase percentages are not affected. Since it has been reported by others (Lettré and Albrecht, 1951) that the antagonistic effect of ATP on colchicine is exerted within a few hours of administration, a time-sequence experiment seemed indicated. The results of this 20-hour experiment, in which one fifth of the cultures were fixed at 4-hour intervals, are shown in FIGURE 9. In this experiment, at the end of the first four hours, the mitotic coefficients of the groups containing ATP plus colchicine and ATP alone are practically identical, and are very nearly zero (.003 and .004). The controls show the normal figure

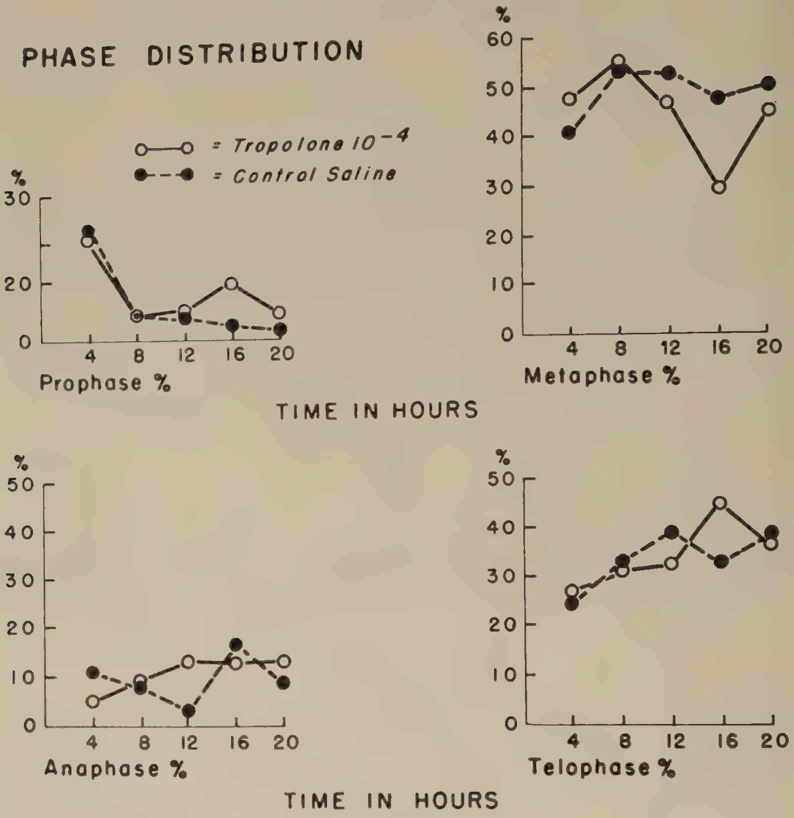

FIGURE 11

of .018, and the colchicine group, as would be expected, is higher (.044). From this point the curve of ATP plus colchicine rises sharply, up to 12 hours, when it levels off as a horizontal line. At no time in this sequence does the mitotic coefficient for ATP alone rise above that of the controls. In ATP alone, the phase percentages are essentially the same as in the controls. The addition of ATP to colchicine does not affect the metaphase arrest (FIGURE 10) though, as shown in FIGURE 9, it importantly reduces the mitotic coefficient. The interpretation seems indicated that the ATP, by bringing about strong contraction of the resting cytoplasm, prolongs the interphase; thus fewer cells enter into mitosis. At the end of 12 hours, when a second mitotic wave should begin, the cell may be nutritionally exhausted. In these observations there is no indication of an effect by ATP on spindle-fiber contraction. The data for this single time-sequence experiment rest on counts of 200,000 cells and 15,200 mitoses.

Tropolone. Analysis of the earlier tropolone experiments in terms of phase percentages gave indication that tropolone influenced the phase distribution in a direction opposite to that induced by colchicine (FIGURE 11). This effect is particularly apparent at about 16 hours. These facts, taken into consideration

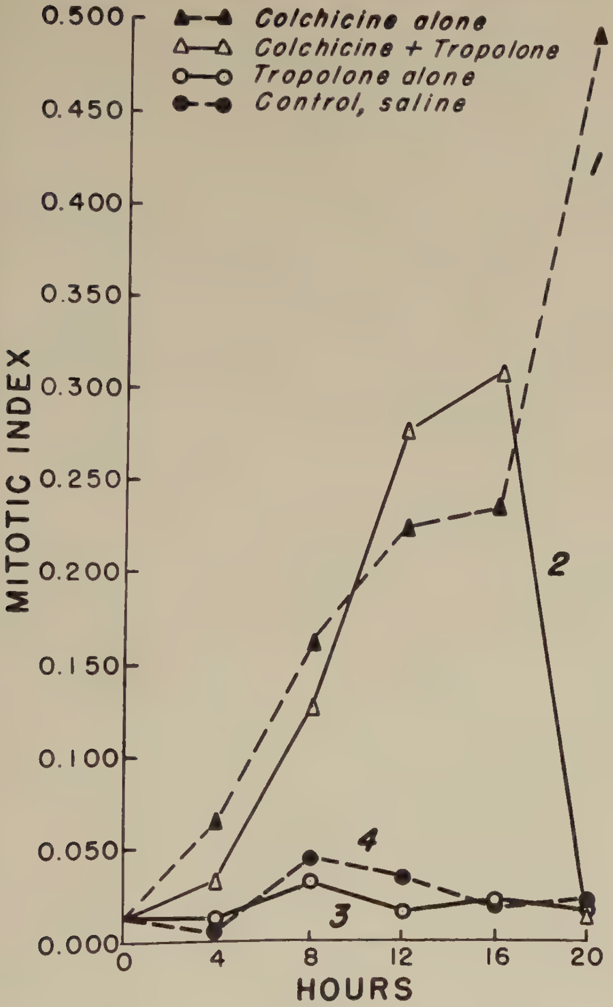

FIGURE 12

with the pattern of the meso-inositol curve, which breaks at about 12 hours, led to the testing of tropolone as a possible colchicine antagonist. Time sequence experiments showed this simple structural analog of colchicine to be its most potent inhibitor, producing a spectacular decline in the mitotic coefficient between 16 and 20 hours after exposure (FIGURE 12). During this time the presence of tropolone with colchicine reduces the percentage of metaphases to 72, a figure halfway between 99 per cent for the colchicine group, and the normal 50 per cent for the saline controls, as noted by Benitez, Murray, and Chargaff (1953). Further analysis indicates that tropolone itself not only shortens the time spent by the cell in mitosis, as compared to the controls, but also shortens the interphase.

Discussion

This antagonism of apparently antimetabolic nature between the two structurally related compounds colchicine and tropolone is of great interest and invites speculation. It is not known whether compounds such as colchicine and tropolone exist in animal cells, but it is known that 7-membered rings exist in plant cells. It may be pertinent that the Syrian hamster, which in its native habitat normally feeds on Colchicum autumnale, is more than five times as resistent to colchicine poisoning as the laboratory rat. With doses of 10 mgm./100 gm. the hamster gains weight and is fertile; at 2 mgm./100 gm. the rat dies of acute colchicine poisoning (Orsini and Pansky, 1952). Development of an ascites tumor of the mouse which is colchicine-resistent also has been reported by Lettré and Kramer (1952). The possibility seems to exist that colchicine may act to produce metaphase arrest by functioning as an antimetabolite in a chain of reactions required for nuclear division.

The evidence gathered within this same biological system lends no support to theories postulating the intervention of ATP in spindle-fiber contraction. When this compound is present in the medium at a concentration of 5×10^{-3}, the cytoplasm of resting cells and dividing cells alike contracts and remains contracted for several hours. Division seems to be prevented by mechanical means from taking place. The internal cohesiveness of the cytoplasm as a whole is decreased by colchicine (Barany and Palis, 1952); ATP, administered along with colchicine, at first restores or even exaggerates the normal viscosity, so that cytoplasmic movement is retarded, and the mitotic coefficient is lower than for the controls. The phase percentages, however, are unaffected; the same percentage of the figures are in metaphase whether ATP is present with colchicine or not. It seems unlikely therefore that ATP is antagonizing the action of colchicine by restoring spindle-fiber contraction. If, as seems demonstrated and agreed, ATP induces over-all contraction of the cytoplasm as a whole, it is difficult to see how this contraction could affect the ordered movement of the chromosomes unless the material of the spindle fiber is presumed to be substantially more sensitive in its response than the general cytoplasm,* or unless the production of ATP in the cell is sharply localized in the spindle area. There seems to be no present evidence that either of these conditions exists.

Though the efficacy and the specificity of meso-inositol in antagonizing colchicine have been repeatedly demonstrated in this biological system over a period of several years, the nature of its action is still obscure. Like ATP, it reduces the mitotic coefficient without greatly changing the phase percentages but, unlike ATP, its effects are delayed until about the beginning of the next mitotic wave. This fact would seem to indicate that it is metabolized. Specific relationship to the spindle remains undemonstrated.

The fact that chemical agents of such diverse nature as the cyclohexene derivative inositol, the ribonucleotide ATP, and the cycloheptene derivative tropolone will reverse in substantial degree the metaphase arrest produced by a single agent—colchicine—confirms the already well-grounded supposition that the mitotic process is chemically an exceedingly complex one. It is also of interest, in this connection, that only one of these agents is able to bring about complete recovery in the presence of colchicine, though they are used at 100-fold its strength, or more. To generalize, one can postulate a variety of routes by which a mitotic poison might operate chemically, as by preventing the production or utilization of an essential metabolite, by interfering with one or more enzymes necessary for the completion of a metabolic process, or indirectly, by depriving the cell of an energy source necessary for one of these processes. The route or routes by which colchicine produces its effects are still unknown, and the methods in which these three agents antagonize them are correspondingly open to conjecture. The results of the experiments described above, however, carried out in a quantitative manner, with a variety of agents and under comparable conditions in the same controlled and standardized biological system, emphasize the dangerous inadequacy of a monistic explanation of mitotic mechanics.

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* According to Weber 1953, concentrations of ATP below 10^{-3} are ineffective for producing contraction in resting-cell models.

Summary

For the general purpose of studying the mode of action of mitosis poisons, the mammalian fibroblast was selected as useful material, and colchicine as a mitotic inhibitor whose effects are fairly clear-cut. To obtain detailed information of a chemical and physiological nature within such a system, three main lines of investigation were followed:

(1) Morphological observation of controls and colchicine-treated tissue from 4 to 72 hours, and establishment of numerical relationships between resting and dividing cells. In treated fibroblast cultures from young male rats, the peak of metaphasic accumulation is reached after 20 hours; this level remains steady for a short time and thereafter declines.

(2) Investigation of other agents, chemically related to colchicine, or reputed to be similar in effect. Among these, tropolone, tropolone methyl-ether, 4,5-tetramethylene tropolone (simple cycloheptene compounds), and gammexane and protoanemonin produce no significant metaphase arrest in this biological system. Aminopterin appears to have a transient effect.

(3) Reversal or antagonism of the colchicine effect by other compounds. Three agents, widely different in chemical structure, have been found to diminish greatly the accumulation of mitoses, when administered simultaneously with colchicine. Meso-inositol performs this function very specifically; *i.e.*, under similar conditions, related compounds such as muco-inositol, inositol lipide, dextro-inositol, epi-inosose, meso-inosose, and common sugars have no such effect. Adenosine triphosphate (ATP) significantly reduces the mitotic coefficient, when employed with colchicine, at five times the lowest concentration found effective by Weber for shortening resting-fibroblast models, but it does not alter the percentage of metaphases arrested. The most potent antagonist of colchicine so far noted is tropolone, one of its simpler structural analogs, which not only produces a spectacular decline in the percentage of metaphases but, when administered alone, shifts the phase percentages in a direction opposite to that induced by colchicine. These findings are thought to cast doubt

on the probability that any single principle will provide an explanation of the mitotic mechanism.

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THE EFFECTS OF CERTAIN STIMULANTS AND DEPRESSANTS ON INDIVIDUAL FIBROBLASTS IN A PERFUSION CHAMBER*

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Knowledge of the chemical, physiological, and morphological relationships of living cells is now more readily attainable because of two research developments: (1) the long-focus oil-immersion phase-contrast system of microscopy; and (2) the perfusion chamber for the growth and maintenance of living cells.

A number of investigators have attempted to supply tissue cultures *in vitro* with a constant flow of nutrient medium. Burrows[1] in 1912 was the first to supply tissue cultures with a constant supply of fresh medium. He used a cotton wick to carry the culture medium through the culture chamber. The fluid drained into a vessel placed lower than the culture chamber. DeHaan[2] in 1928 had an apparatus which made possible the circulation of large volumes of liquid, but the apparatus was too complicated. Carrel[3] in 1931 designed a pump flask for the continuous circulation of a fluid medium. This device was improved by the help of Lindbergh.[4] Even though markedly better, in that attention was paid to the gas tension of the fluid, the apparatus did not allow for adequate observation of the cells. Pomerat[5] uses a very simple perfusion chamber which allows observation under the best modern optical conditions, but which has the disadvantage of being temporary and requiring constant attention. His apparatus does not provide for maintenance of constant gas tensions, and it is not well adapted for prolonged observations. Christiansen, Danes, Allen and Leinfelder[6] modified the Pomerat chamber and made it a permanent piece of apparatus. They standardized the dimensions and changed the medium with syringes. Their device gives control of the medium from both sides of the chamber and, if desired, allows the medium to flow through the chamber. To oxygenate the culture, the fluid is periodically replaced by air. But their apparatus is not automatic, and the equilibrium with air is not satisfactory.

The conventional method for studying the effects of drugs on living cells *in vitro* has been to incorporate the drug into the medium in which the cells are explanted and grown. The disadvantages of this method are: (1) the immediate responses of the cells cannot be observed because it requires several hours before the first cells have grown out from the explant; (2) the concentration of the drug cannot be maintained; (3) the drug cannot easily be removed; (4) the effects cannot be observed on a single cell over the entire experimental period; and (5) repeated doses cannot be given to the same cell. To correct these difficulties, we have devised a general method for studying living cells under a variety of known controlled conditions. The perfusion method reported here

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† The data on the effects of drugs on cells is abstracted from a dissertation submitted by the junior author for the Ph.D. degree at the University of Pittsburg, 1954.

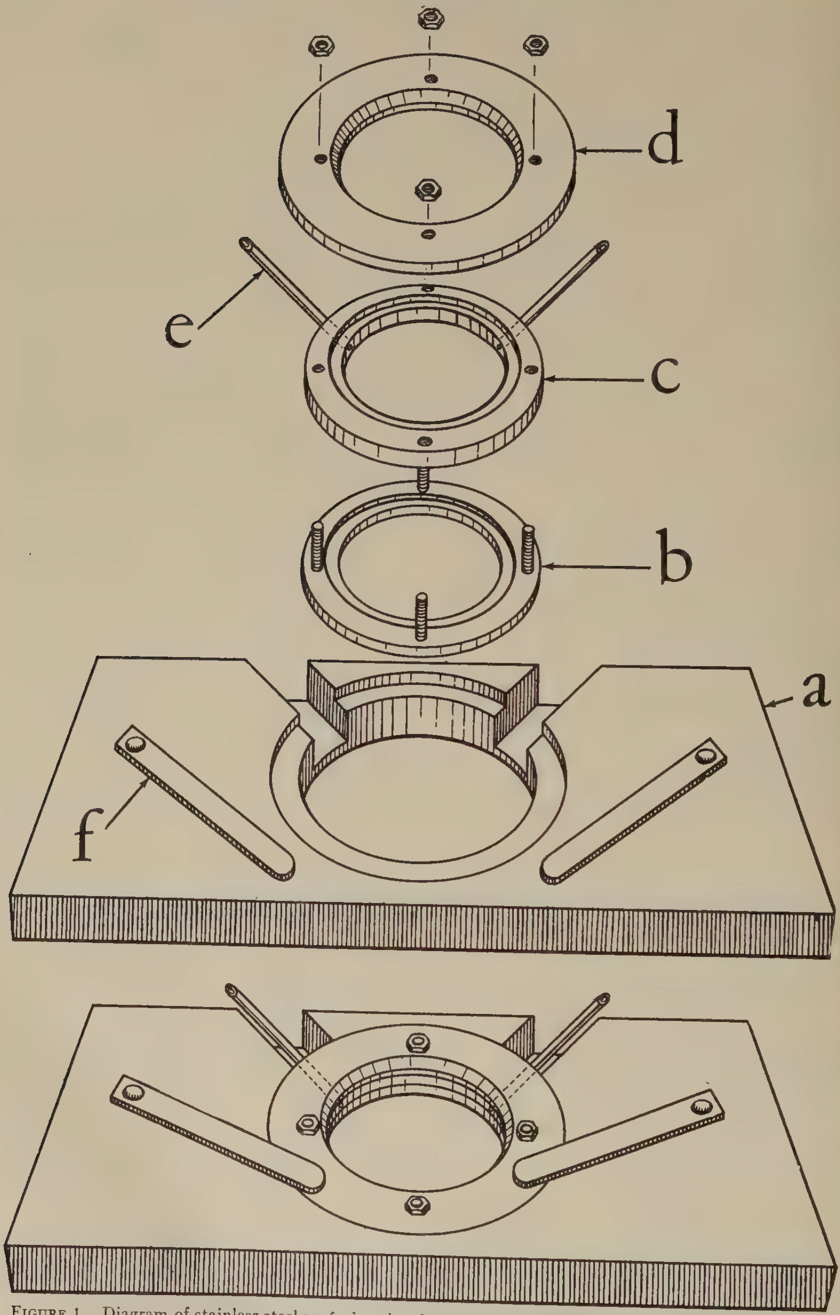

FIGURE 1. Diagram of stainless steel perfusion chamber. *Below*, assembled; *above*, exploded view. *a*, 75 × 39 × 3 mm. slide; *b*, lower ring; *c*, middle ring bearing in-flow and out-flow No. 19 stainless steel tubing; *d*, upper retaining ring; *e*, No. 19 stainless steel tubing; *f*, leaf spring clips.

embodies improvements which overcome the difficulties stated in regard to conventional methods for studying cells *in vitro*, particularly for studying the effects of chemical agents on cells *in vitro*.

Methods. The perfusion chamber used in this study is shown in FIGURE 1. It consists of a stainless steel slide, *a*, 75 × 39 × 3 mm., machined as shown, to receive a circular inserted chamber which holds the culture. The chamber is shown assembled in the lower part of the figure; and in exploded view in the upper part of the figure. The cover glasses are not shown. The chamber is assembled by placing a circular coverglass (22 mm. in diameter) into the recessed center of part *b* (the coverslip is sealed into place with a little warm beeswax). Then part *c* (bearing the No. 19 gauge stainless steel tubing *e*) is slipped over the upright screws. The coverslip bearing the culture is put face down in the recessed area in *c*, and the retaining ring *d* is secured with nuts to hold the culture-coverslip firmly in place (again, a little beeswax provides a better seal). The circular perfusion chamber is held onto the slide *a* with leaf springs *f*. After the whole is placed on the microscope stage, polyethelene tubing (No. 200) is connected to the steel tubing attached to the chamber. The microscope is kept in a hot box, which is maintained at 38° C. The lucite hot box, FIGURE 2, is heated with hot air forced through flexible automobile-heater tubing from a small power blower-assembly, *C*. The hot box consists of two parts: part *A* contains incurrent and excurrent connections for the recirculated hot air and the thermostat, and is fixed to a base; part *B* is removable and allows free access to the microscope during the time the apparatus is set up.

The normal nutrient medium is forced by gas pressure through the chamber from a pyrex glass perfusion line shown in FIGURE 3. The components are provided with interchangeable ground-glass ball-and-socket joints. A two-stage, automatic, gas pressure-regulator *c* provides for reduction in pressure of a gas mixture consisting of 95 per cent oxygen and 5 per cent CO_2 from tank *a*.

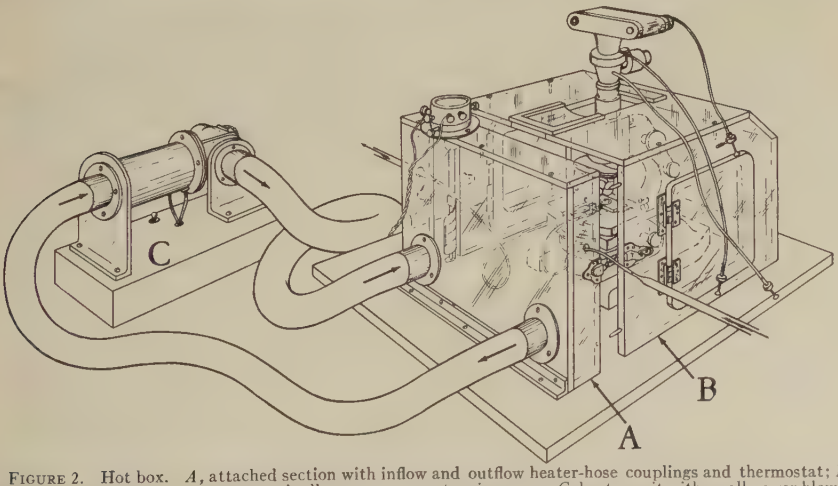

FIGURE 2. Hot box. *A*, attached section with inflow and outflow heater-hose couplings and thermostat; *B*, removable section which, when removed, allows easy access to microscope; *C*, heater unit with small power blower operated by thermostat in section *A*. The hot box may be made of lucite.

A needle valve *b* and a micro-regulator pinchcock *d* is inserted on the line for a more critical adjustment of the gas flow. Various gas mixtures may be substituted for other experimental purposes, of course. The gas is washed and saturated with water *e* and filtered through a fritted glass medium porosity bacterial filter *f*. The rate of gas flow is maintained constant by means of the column of water *g* open to the line through *h*. The pressure of the gas may be measured in mm. of water (or other fluid) in a manometer *j*. The usual pressure, adjusted by changing the level of the water in *g*, was approximately 15 cm. of water above atmospheric pressure. The gas mixture bubbles through the medium in *m* and escapes continuously through a minute vent, *n*. This provides for continuous equilibration between the gas and the medium; it also adjusts the system to changes in barometric pressure. The medium leaves the flask and enters the perfusion chamber through a 1-mm. capillary line. Samples of the fluid may be taken before it enters the culture chamber through stopcock *r*, and after it leaves the culture chamber through stopcock *y*. Any difference between these two samples is a measure of what happened in the culture chamber. The perfusate flows from the chamber into reservoir *z*. An experimental medium from line *q* may be quickly substituted for the control medium by manipulating stopcocks *k* and *p*.

In this study, the stock cultures were derived from the periosteum of the long bones of 7-to 9-day old chick embryos in a medium of chicken plasma and chick embryo extract. Subcultures were made on 22-mm. No. 0 circular coverslips by the double coverslip method. After 24 hours, the coverslip bearing the culture was placed in the perfusion chamber as described above. The control medium in the perfusion system consisted of 25 per cent horse serum and 75

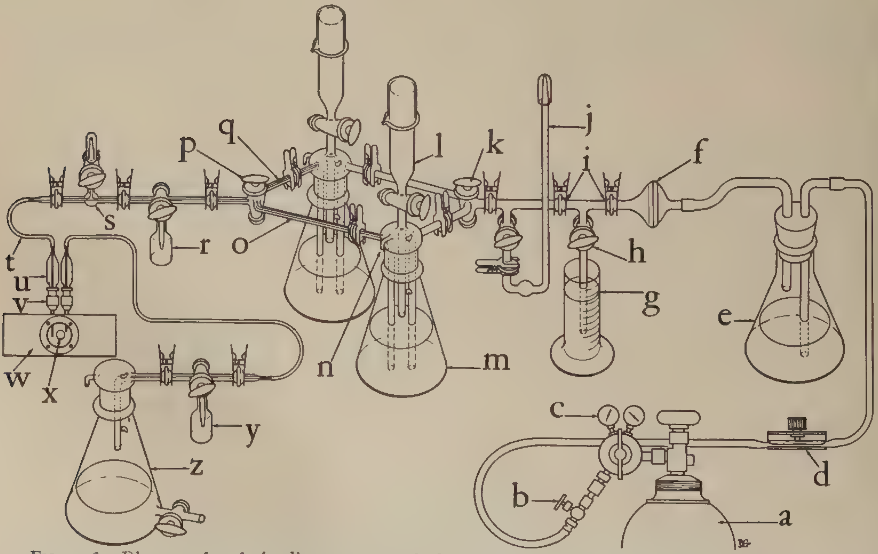

FIGURE 3. Diagram of perfusion line. *a*, gas tank; *b*, reducing valve; *c*, automatic reducing valve; *d*, micro pinchclamp; *e*, washing bottle; *f*, bacterial filter; *g*, *h*, *i*, pressure regulating device; *j*, manometer; *k*, *m*, *o*, *p*, one line; *k*, *q*, *p*, alternate line; *l*, filling device; *n*, gas escape vent; *r*, sampling cock; *s*, bubble-escape cock; *t*, *u*, *v*, intake line; *y*, sampling cock; *z*, exhaust reservoir.

per cent Tyrode's solution. The experimental medium consisted of the control medium, to which was added the drug to be studied. The same molarity (4.4×10^{-3} M) was used for all drugs initially in order to establish some basis for comparison. This value was chosen somewhat arbitrarily for convenience and has no theoretical significance. Each experimental medium was perfused for 5 to 20 minutes over several cultures, and the cells were photographed. Each type of response of a cell to a drug is referred to as a "cytosyndrome." Among the drugs studied were the following: pentobarbital sodium 1.0 mgm./ml.; pentothal sodium 1.3 mgm./ml.; phenobarbital 1.0 mgm./ml.; barbituric acid 0.25 mgm./ml.; morphine sulfate 3.3, 1.0, and 0.25 mgm./ml; codeine sulfate 3.7 mgm./ml.; strychnine 3.8, 2.8, 1.4, 0.5 mgm./ml.;

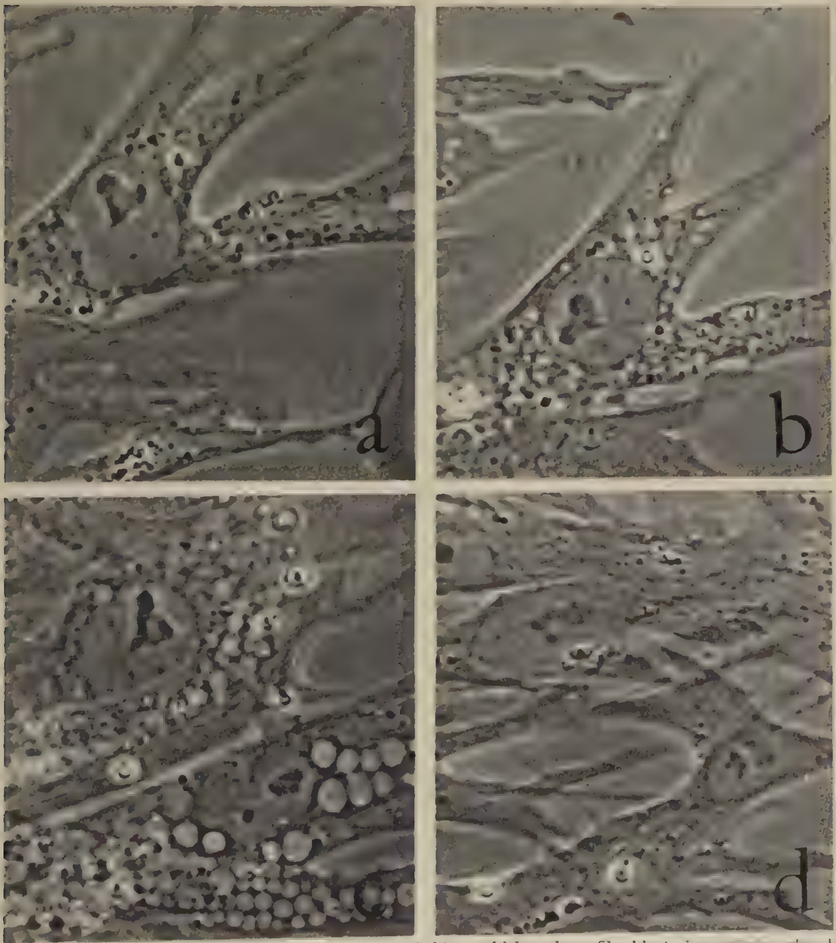

FIGURE 4. Action of morphine sulfate (3.3 mgm./ml.) on chick embryo fibroblasts in perfusion chamber. Phase contrast photomicrography; nuclei are about 10 μ in diameter. *a*, normal cell spread out on surface of coverglass with typical long filamentous mitochondria. *b*, after treatment, vacuoles formed, filamentous mitochondria present, cytoplasm still extended. *c*, cell with very large vacuoles, vacuoles continue to form and coalesce long after drug is washed away. *d*, 5 hours after beginning of perfusion; cells essentially normal.

benzadrine sulfate 1.0 and 0.4 mgm. ml.; and adrenalin chloride 2.0 mgm. ml. Many other substances could have been used, and a few preliminary runs were made on a variety of substances.

It might be thought that cells would respond to all toxic substances in a rather similar manner; however, we found that there were a number of very different cytosyndromes. Before discussing these reactions, we shall describe separately the cell responses to a few drugs. Each drug was allowed to act until an obvious visible response occurred. Then the drug was slowly flushed out with the control medium. All of the cell changes we are concerned with in this study were reversible, that is, the cell returned to its normal appearance some time after the drug was removed.

Pentobarbital sodium. After about 15 minutes of perfusion, long protoplasmic extensions of the cell were withdrawn toward the cell. Small rounded pulsating

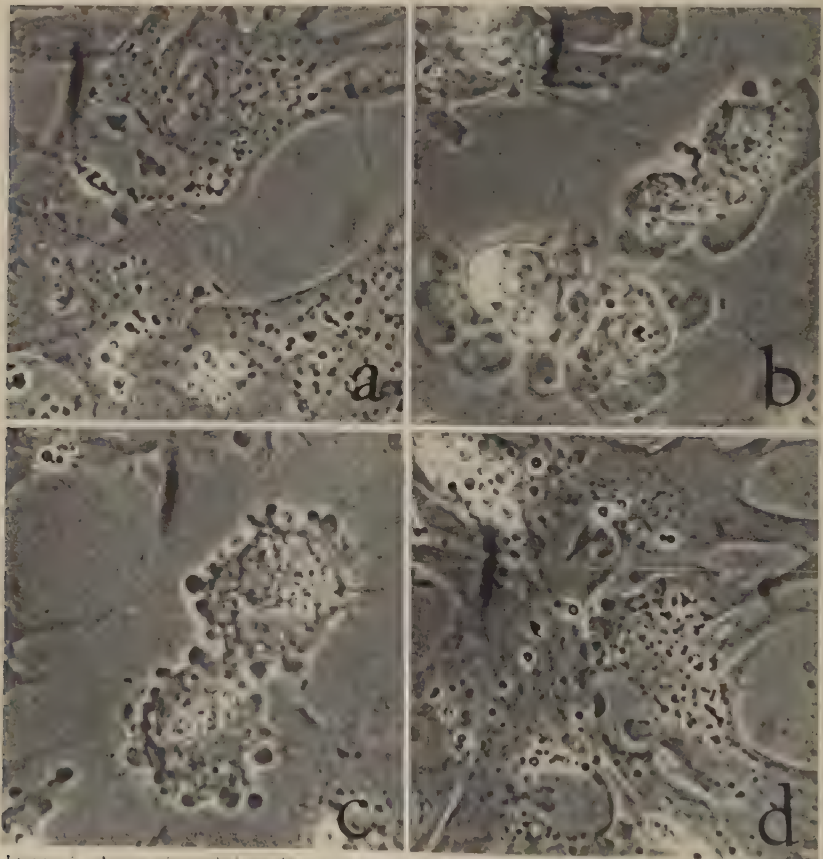

FIGURE 5. Action of strychnine sulfate (3.8 mgm./ml.) on chick embryo fibroblasts in perfusion chamber. Phase contrast photomicrography; diameter of nucleus about 10 μ. *a*, normal cells spread out on coverglass. *b*, six minutes after perfusate has reached cells the cytoplasm has withdrawn and a "boiling action" takes place. *c*, the strychnine was removed after 10 minutes and the cells began to recover. Photograph made 35 minutes after beginning of perfusion with drug. *d*, 80 minutes after the strychnine was removed, the cells have recovered so that they were indistinguishable from normal.

blebs appeared and disappeared along the cell membrane. The filamentous mitochondria were shortened and thickened and reduced in number. No change was seen in the appearance of the nucleus. Complete recovery was apparent about one hour after the drug was removed.

Morphine sulfate. There was little shrinkage and almost no bleb formation. Small vacuoles gradually appeared, and after about 15 minutes the cell contained a great many vacuoles which were small at first and gradually increased in size. Granules which constantly moved about were present within the vacuoles. Some time after the drug was flushed out, the vacuoles continued to increase in size; then they coalesced and gradually disappeared. Complete recovery was apparent about two hours after the drug was removed. Some of these changes are shown in FIGURE 4. As might be expected, at 24° C., the whole cytosyndrome was slowed.

Strychnine sulfate. The normally outstretched cell rapidly withdrew its cell processes and underwent a violent "boiling" action, which involved the entire cell; this condition reminded us of the appearance of a cell about to divide. The time-lag for the first visible response at a concentration of 4.4×10^{-3} M was only a few minutes after the beginning of perfusion; the drug was perfused for 10 minutes. By reducing this dosage to one fourth, a much less violent but similar cytosyndrome was produced. Complete recovery was apparent about one hour after the drug was removed. If the drug was again perfused through the preparation the same result was observed.

The action of pentothal sodium, phenobarbital, barbituric acid, and adrenalin chloride was similar to that produced by pentobarbital sodium. The action of codeine sulfate and benzadrine sulfate, and very dilute doses of strychnine sulfate produced vacuoles similar to, but fewer in number, than those produced by morphine sulfate (FIGURE 5).

In general, the degree of response of the cells is influenced by the concentration of the perfused chemicals as well as the length of time they surround the cells and are permitted to act on them. Repeated small doses, with sufficient time allowed for recovery between doses, do not produce the same picture as a single large dose.

Discussion

It is too early in this series of observations to attempt to develop a general theory of the mechanism of action of drugs on living cells under these conditions. But enough has been done already to show that the method described here has certain advantages over other methods. The principal advantage is that morphological changes can be seen during the entire period of experimentation and the cells are kept at an optimum condition. In the whole organism, the net effect of the action of a drug is usually to cause the whole body to respond in some way through the action by the drug on one or at most a few tissues. Thus the action on many cells may be obscured. Or the animal may die because a drug stops respiration or interferes with some other vital function, even though the majority of the cells in the body are not irreversibly adversely affected. The intimate nature of exactly what is happening at a cytochemical level is, of course, difficult to ascertain primarily because of the small amount and low concentration of substances in the perfusate. This concentration and amount may be increased somewhat by slowing the rate of flow of the perfusate. However, there is a limit to this retardation because the slower the rate of flow, the less the effective rate of supply of oxygen and nutrients and the removal of waste products. It is the ultimate hope that there can be found one-to-one correspondences between the chemistry of the medium and visual responses of the cell—and it may be that we are on the threshold of such a situation.

Summary

A method is described for studying the effects of chemical substances on living cells in a perfusion chamber which permits the automatic continuous flow at known rates and under known gas tensions of liquid media past the cell under observation. The medium can be changed at any time without interrupting the continuous observation of the cell. A number of depressants and stimulants were studied, and their effects were described and recorded by time-lapse phase cinephotomicrography. A limited number of cytosyndromes (patterns of cell behavior) were distinguished.

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QUANTITATIVE CINE ANALYSIS OF CELL ORGANOID ACTIVITY*

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Tissue Culture Laboratory, University of Texas Medical Branch, Galveston, Texas

Recent improvements in the technique for managing cells *in vitro*, together with a growing appreciation of the value of phase-contrast, time-lapse cinematography in the development of dynamic concepts regarding human elements, invite precise studies on drug action. Such operations can be facilitated with the use of perfusion chambers of the types described by Pomerat,[1] Christiansen, Danes, Allen and Leinfelder,[2] and Buchsbaum, Wertman, Dwyer, Kuntz, and Gillette.[3]

In his "Cell Physiology and Pharmacology", Danielli[4] has presented important suggestions for attacks on the problem of the mechanism of drug action at the cellular level. For the tissue culturist concerned with behavioral activities which can be recorded with time-lapse photography, it becomes necessary not only to be thoroughly familiar with the mobile structures themselves, but also to evolve techniques by which the data available in the dynamic film projection can be converted to the static printed page by using reproductions of selected film frames. Of even greater importance is the necessity for evolving simple, reliable methods for converting positional changes of cell organoids into graphs from which generalized formulas or constants may be extracted. A method which would make possible an accurate evaluation of membrane activity associated with pinocytosis (cell drinking) might be of inestimable value in describing the action of surface-active agents such as detergents. A better appreciation of factors regulating the speed of cell intake could prove of practical value in reducing or increasing the ingestion rate of components introduced into the culture medium. Such information would enrich our knowledge of cell nutrition and of drug action.

Frederic[5] and his associate have made important inroads in the analysis of changes in mitochondrial movement and form. Because their contributions in this field are represented elsewhere in this monograph we have omitted discussion of mitochondria *per se* in this article. However, some overlap of statements will be found with those in a report[6] made at the Lisbon meetings of the Société Internationale de Chirurgie where some of the same ground was covered and certain essential bibliographical references were noted. The present paper is concerned particularly with the mechanics of analysis and the reproduction of new data.

General Considerations

Thorough familiarity with important film sequences can be enhanced by repeatedly viewing the images in reverse order. For this purpose film viewers or projectors of the type represented by the Kodascope film analyst are especially valuable. Manual control of the rate of speed afforded by a well constructed film viewer is highly desirable. Greatly exaggerated changes in projection rate may reveal rhythmic phenomena not clearly manifest at the two or three standard time-lapse rates generally employed in preparing film sequences. Indeed, it is considered advisable to build a film collection with recordings at a limited number of speeds, such as 1, 4, 8, and 16 frames per minute. Under such conditions the investigator learns to judge cellular events against relatively few time-accelerated constants. Having become familiar with the activity of such organoids as mitochondria under these conditions, the film viewer can then be used with special advantage to reinspect other temporal relations.

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* This study was aided by a contract between the Office of Naval Research, Department of the U. S. Navy, and the University of Texas, NR 131-163.

After isolating a particularly critical portion of the film, it is often valuable to print the entire section. If a clock is not used to record the exact time directly on the film, the emulsion at the margin may be scraped and reference numbers written with India ink. For convenience, sheets of 8 in. × 10 in. paper are cut into four strips of 2 in. × 10 in., on which four sets of 10 frames from 16-mm. film can be enlarged to a size convenient for study. By means of Scotch tape the paper strips, arranged in deciles, are attached face down to a piece of window glass so that they can be examined through it. By means of china-marking pencils, the position of particular structures, or of significant stages, can be checked on the glass. Such marks are easily changed until ideal frames are selected. Frequently, it is desirable to enlarge portions of the film which represent critical events. A series of from four to eight such frames to conform to the size of journal page plates does not require enlargements beyond what can be reasonably expected to give considerable detail without undue interference from emulsion grain. It is practical to make enlargements of individual 16-mm. film frames to 4 in. × 5 in. size using an intermediate Isopan negative, which reverses the image and remains available in a reference film-negative file.

Illustrative Examples of Analyses

(1) *Nuclear rotation.* With large well-defined structures it is possible to follow the simple expedient of projecting the film from a standard enlarger onto architect's tracing paper. Pencil tracings, reinforced if necessary with India ink, are useful for subsequent study. An ideal case for this treatment is illustrated in the analysis of nuclear rotation in cells from the human nasal mucosa.[7] Since the nuclei of epithelial elements often contain two nucleoli, and because the rotatory activity is practically "on the flat," the angle of rotation can be read with a protractor from a pencil line drawn through the nucleoli, the midpoint between these serving as the axis of rotation.

Nuclear rotation has been observed repeatedly in cultures of epithelia. FIGURES 1 to 10 illustrate rotatory activity in a cell from a culture of a cervical carcinoma (HeLa) which was isolated about four years ago in the laboratory of Doctor George Gey. Both clockwise and counter-clockwise movements of the nucleus have been recorded. The mechanism involved and the physiological significance of this phenomenon is under study.

(2) *Muscular contractions.* While the rhythmic contractions of chick-heart

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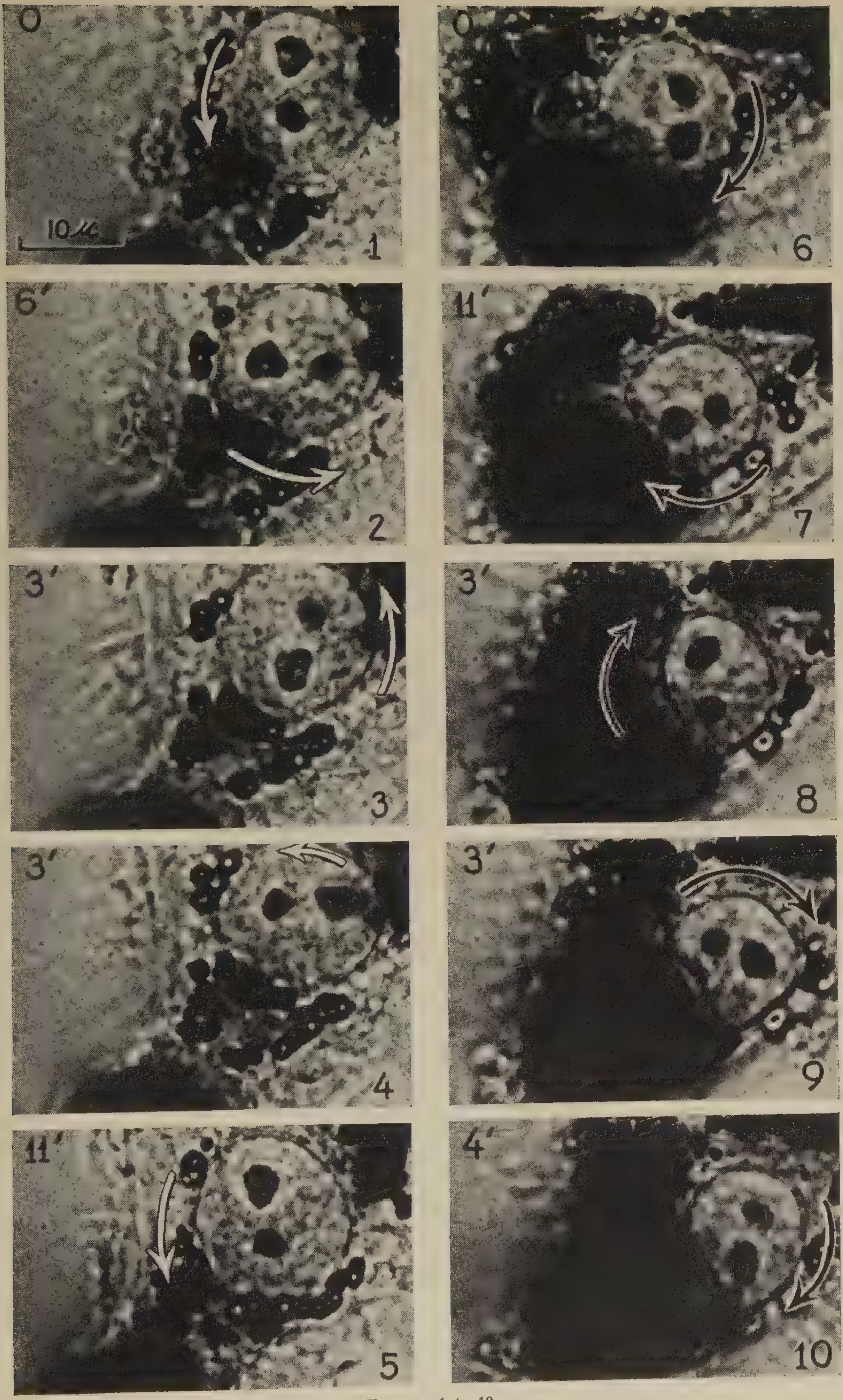
FIGURES 1 to 10. Nuclear rotation in a cell from a cervical carcinoma (HeLa strain) in long-term culture. Time intervals between selected frames from a cinematographic record are given in the upper left quadrant of each photograph. Arrows indicate the direction of rotation for counterclockwise (FIGURES 1 to 5) and clockwise movements (FIGURES 6 to 10) of the same nucleus. The magnification is indicated on FIGURE 1.



Figures 1 to 10.

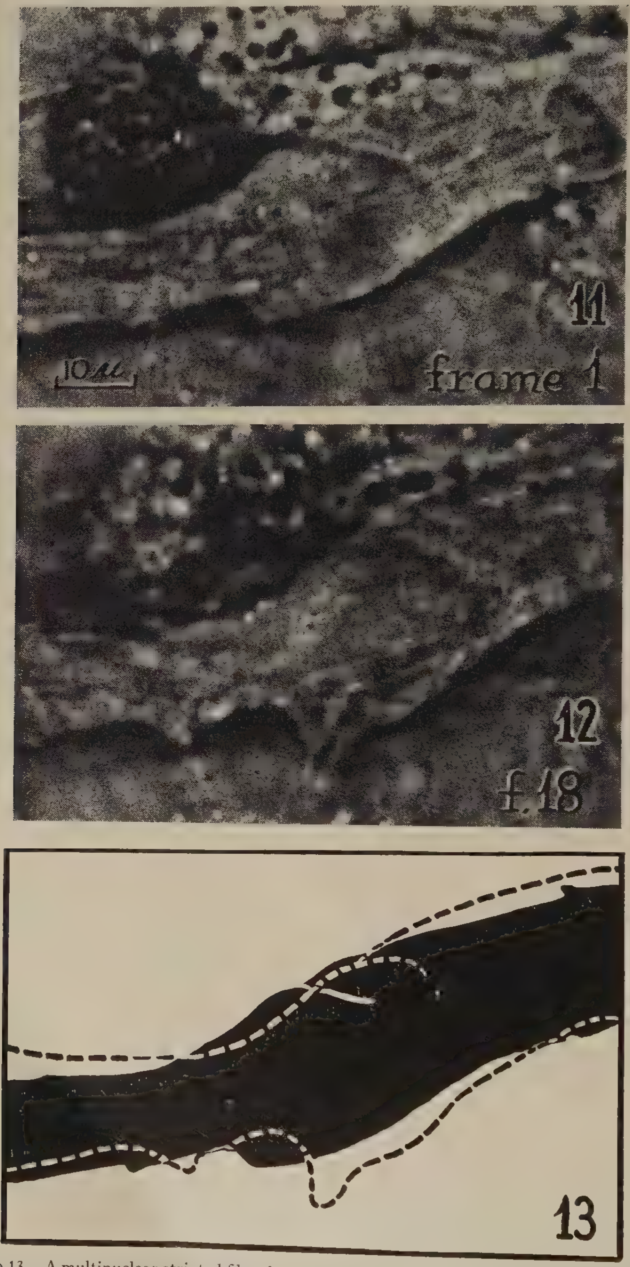


Figure 11 to 13. A multinuclear striated fiber from a 12-day chick-embryo leg muscle in culture for five days. The 1st frame (relaxation) and 18th frame (contraction) selected from a film record taken at 16 frames per second are shown with a diagram indicating alterations in the form and position of the fiber in this field. Note changes in density in the sarcomere bands. The magnification is indicated in figure 11.

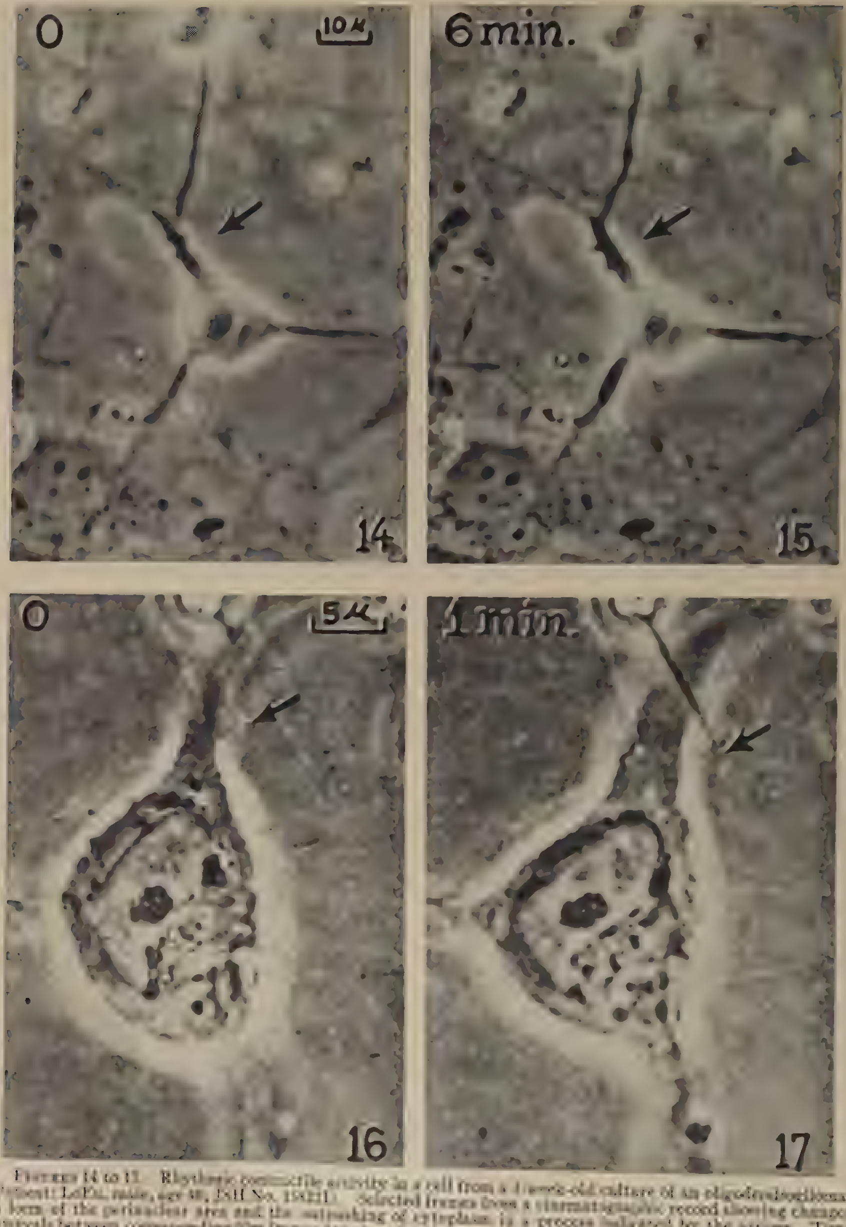
explants can easily be counted by direct observation on a warm stage with the use of a stop watch,[8] more elegant records may be executed with an ordinary Grass EEG instrument as recently described by Diserens.[9, 10] It is hoped that similar quantitative studies will soon be available for the description of rhythmic activity of isolated skeletal muscle fibers from chick embryos.[11]

Bennett and Porter,[12] using the technique of electron microscopy, found evidence for the conclusion that increase in density and osmium binding capacity of all sarcomere bands during contraction might be associated with the movement of sarcoplasmic material into the myofibril, and that the Z and M bands may serve as points of entrance. FIGURES 11 to 13 demonstrate changes in sarcomere bands during contraction in living preparations of 5-day-chick leg muscle as revealed with dark phase contrast optics. Work in process is designed to add information regarding this phenomenon, especially in relation to drugs known to affect the biochemical events occurring during contraction.

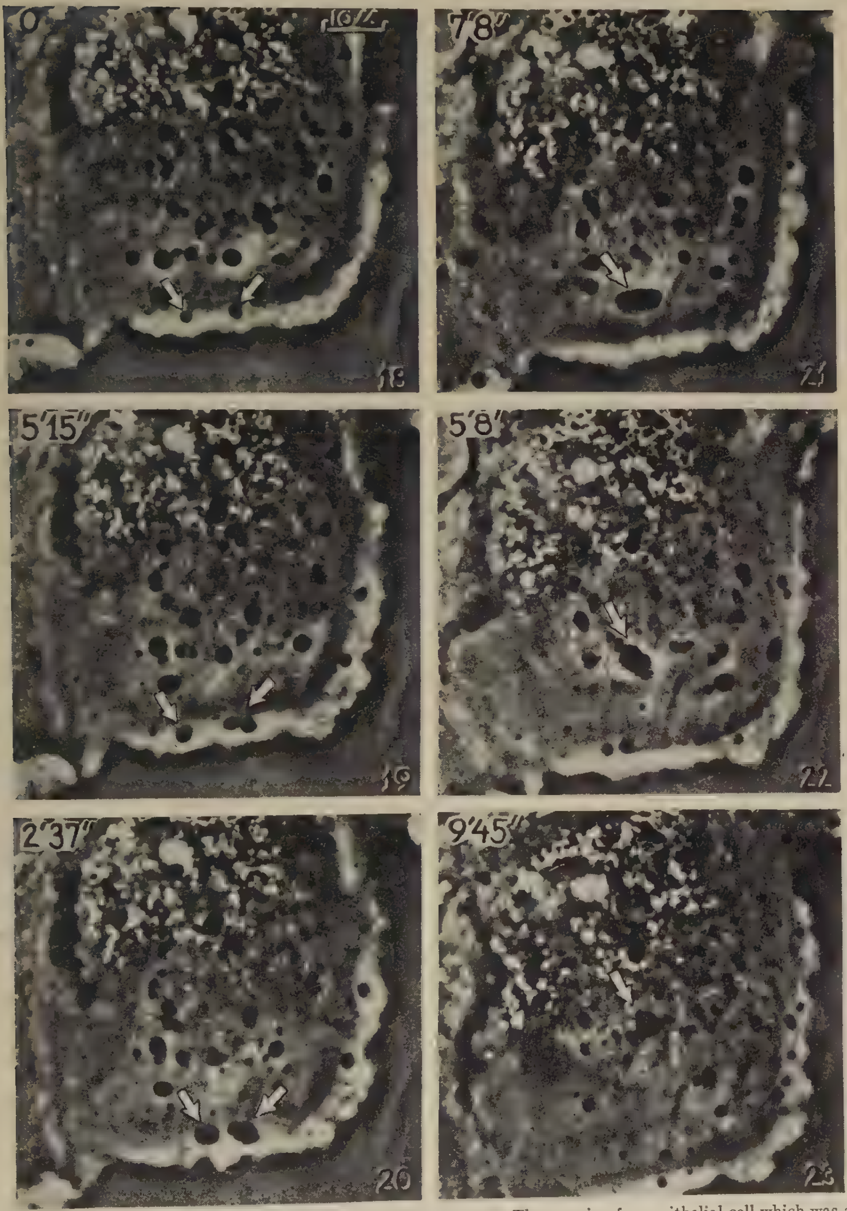
(3) *Pulsatile activity of oligodendroglia.* After several years' quest, the opportunity to cultivate cells from an oligodendroglioma, as had been done originally by Canti, Bland, and Russell[13] was realized. With the use of both an 8 mm. and a 1.9 mm. phase-contrast objective, records were made from which the cycle of contractions could be worked out. The frequency of these movements was found to be of the same order as those reported for oligodendroglia from the corpus callosum of the normal rat[14] and from various human sources.[15] The results of this study will be published *in extenso* elsewhere. For the purposes of the present report, FIGURES 14 to 17 show form changes between the expanded and contracted states which are especially well reflected by the diameter of the cytoplasm in the process indicated by arrows. From stages such as these, which can be more clearly understood in the actual film projection, a graph is being prepared to describe temporal relations of the contractions. Here again it is hoped that the evolution of a sufficiently sensitive recording instrument ultimately will permit the making of synchronous film and action current records under controlled and experimental conditions. Such methods are an essential "next step" in the study of human brain cytophysiology.[16]

(4) *Pinocytosis.* In planning pharmacological studies on human cells in tissue culture, tonsilar tissue deserves high consideration as a rich source of test material. This tissue, which easily can be obtained in quantity from young individuals without the complication of acute inflammatory states, shows a marked capacity for emigration and for mitotic activity in a thin epithelial sheet. However, much further work needs to be done on its biology. Only five references on the growth of the tonsil *in vitro* are cited in Murray and Kopech's *Bibliography of the Research in Tissue Culture* (1953). Kelemen[17] found that tonsil tissue suppressed the outgrowth of chick explants in conjoint cultures. In our own experience, limited observation has revealed a relatively large number of multipolar mitotic figures. In addition to these interesting leads, for the purposes of this study, tonsilar epithelium provided an exceptionally fine example of cell drinking as shown in FIGURES 18 to 23.

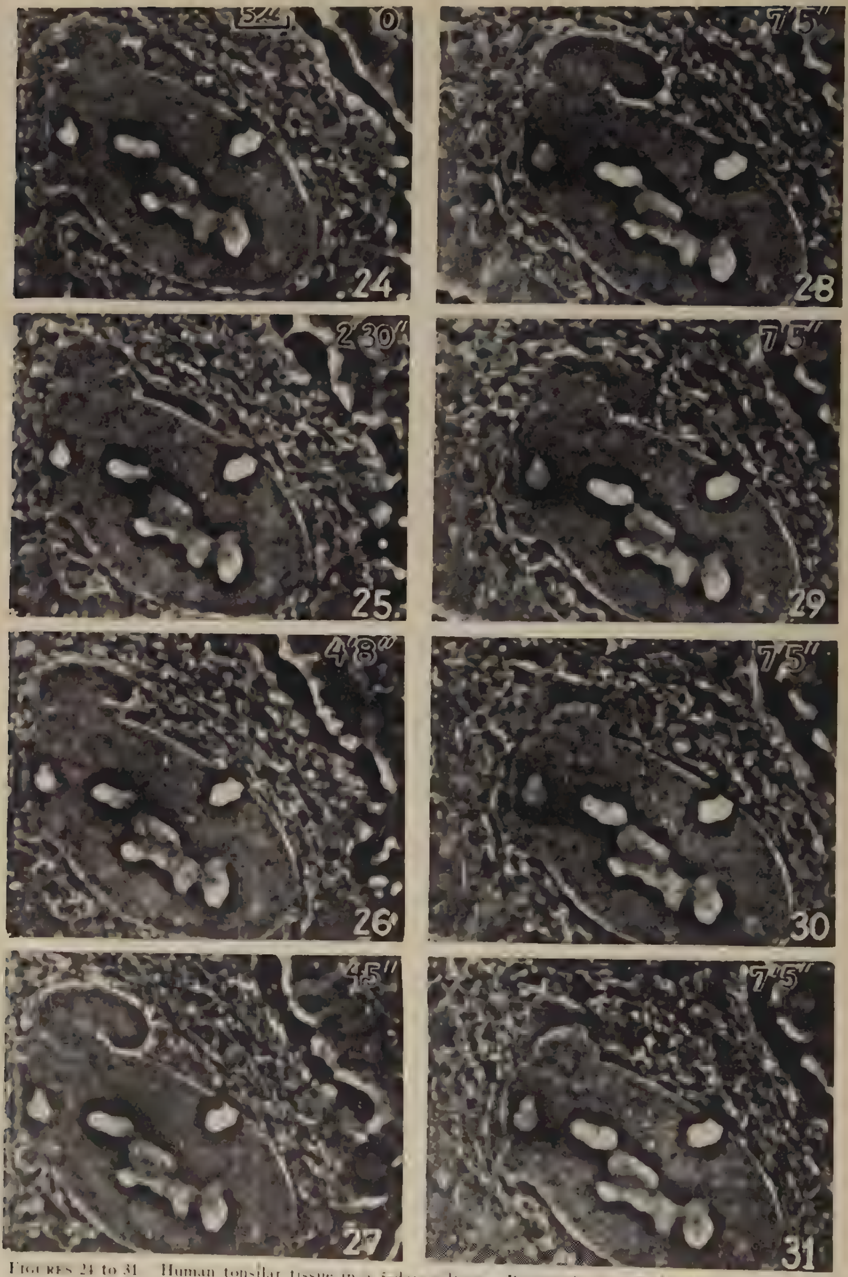
Lewis[18] estimated that the total volume of vacuoles accumulated in one hour may be equal to one third the volume of the cell. It seems incredible that this



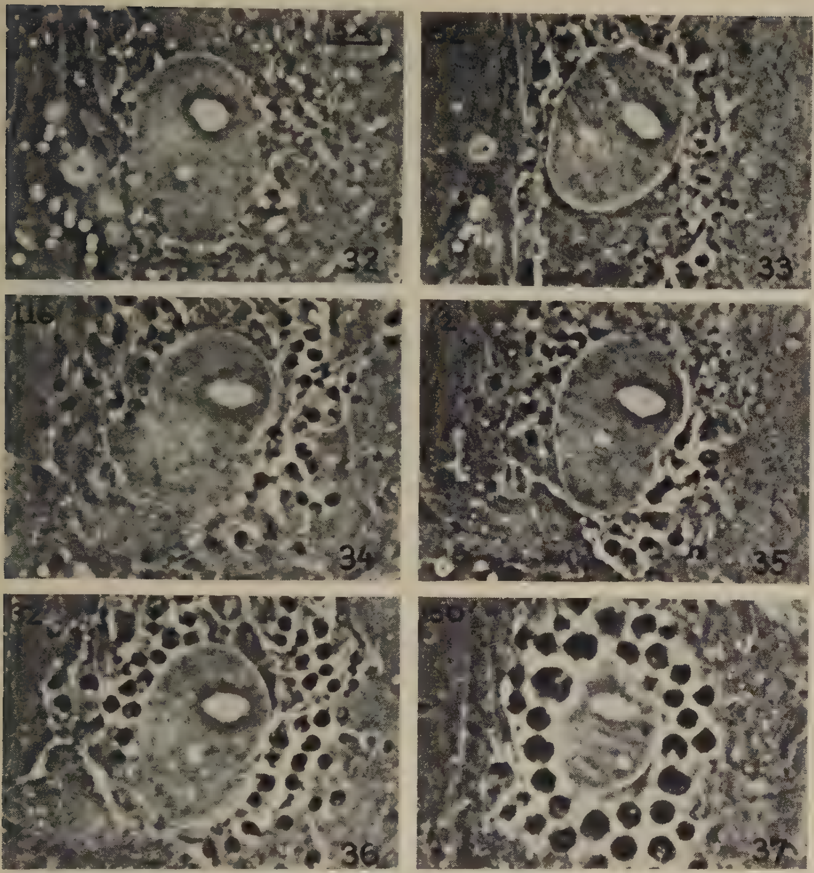
FIGURES 14 to 17. Rhythmic contractile activity in a cell from a 4-week-old culture of an oligodendroglioma. (Patient: [illegible], male, age 48, [illegible] No. [illegible].) Selected frames from a cinematographic record showing changes in form of the perinuclear area and the outpushing of cytoplasm in a process indicated by the arrows. Time intervals between corresponding film frames are given in minutes and the magnification is indicated on the initial photograph for each of the two series.



FIGURES 18 to 23. Human tonsilar tissue in a 5-day culture. The margin of an epithelial cell which was actively engaged in pinocytosis (cell drinking). Arrows indicate the formation of a particularly large vacuole (FIGURE 21) which migrated toward the nucleus and showed the beginning of fragmentation (FIGURE 23). Time intervals between successive frames are given in the upper left quadrant of the photograph. The magnification is indicated on FIGURE 18.



FIGURES 24 to 31. Human tonsilar tissue in a 5-day culture. Progressive herniation and recovery of the nuclear membrane suggestive of a contribution to the cytoplasm from the nucleus is indicated in the upper left quadrant of selected frames from the cine record. Time intervals between individual pictures are given in the upper right area of each print and the magnification for the series is shown on FIGURE 24.



FIGURES 32 to 37. The effect of atropine sulphate on a living cell from the adult human nasal mucosa. The drug was introduced in a perfusion chamber containing a 5-day culture prepared from a biopsy of tissue from the middle turbinate. The appearance of the cell immediately before the addition of the drug is shown in FIGURE 32. Note the presence of slender filamentous mitochondria. In FIGURE 33, taken 62 minutes after the addition of 200 γ/ml. of atropine, the mitochondria showed a tendency to clump and fuse around newly formed vacuoles. These processes became more conspicuous with time, as shown in FIGURE 34, which was made 116 minutes after the introduction of the atropine. FIGURE 35 represents the same cell one-half minute after the addition of 500 γ/ml. of atropine sulphate. This concentration of the drug is seen to have produced an exaggeration of mitochondrial fusion and of vacuolar formation after 32 minutes (FIGURE 36) and after a total of 182 minutes (FIGURE 37). The magnification for the series is indicated on FIGURE 32. The record was made with bright phase-contrast optics.

important phenomenon has been almost totally neglected by cell physiologists, and that Lewis is the only investigator employing the tissue culture method who has attempted to explore its mechanism.

(5) *The mitotic phenomenon.* The analysis of the temporal changes in chromosomes and other bodies which move during cell division constitutes an enormous chapter which lies outside the scope of this report. Reference to the recent book by Hughes[19] will serve as an invaluable source for many of the possible avenues of inquiry available to the pharmacologically-minded cytologist in this area of investigation.

(6) *Activities in the metabolic nucleus.* Among the changes observed in nu-

clear form, herniation of the nuclear membrane from a record of human tonsilar epithelium is shown in FIGURES 24 to 31. This phenomenon, seen to be repeated many times, invites the speculation that products may have been extruded from the nucleus but much further study would be necessary to justify such a conclusion. However, current trends concerning the possibility that some synthesis proceeds from the intranuclear situation should alert workers utilizing the tissue culture method to look for morphological evidences of activity in this region. Such indications have been accumulating in our library, which now comprises perhaps 70,000 feet of 16-mm. film. Particularly significant are rapidly changing spots in the density of the nucleolus of a nerve cell from the paraventricular nucleus, and in the contour of the nuclear membrane adjacent to the juxtanuclear zone of infected fetal brain cells treated with an immune body.

(7) *Changes in the Juxtanuclear Zone.* Analysis of activities of this dynamic center is complicated by the variety of structures, the complexity of movement, and the optical density in the area.

The convergence upon and disappearance of droplets in the juxtanuclear zone, which are accreted in the course of pinocytosis, was illustrated from film records by Pomerat[6] for cells from the human conjunctiva. Spherules appear to fragment, or reduce in size, very rapidly as they approach the nuclear membrane. Some records are reminiscent of observations on the activity of the contractile vacuoles in amoebae. Stages showing the rapid disappearance of vacuoles in a cell of astrocytic type from the spinal cord of an adult cat have been published.[16]

Preliminary experiments on the effect of atropine on cells cultured from the adult human nasal mucosa have been performed in a perfusion chamber and recorded with ciné technique. Selected frames (FIGURES 32 to 37) show progressive fusion of mitochondria and the formation of conspicuous vacuoles.

Discussion and Summary

The method of determining the relative concentration of labeled nucleic acid precursers by neoplastic and embryonic cells, as reported by Fitzgerald,[20] and a series of procedures for the study of cell components with interference and ultraviolet microscopy described by Mellors[21] give recent evidence of the importance of tissue-culture preparations for quantitative cytological analyses. To these attacks belong the study of phase-contrast time-lapse cinematographic records of living cells. Suggestions have been made regarding general procedures and special techniques followed in the analysis of (1) nuclear rotation, (2) muscular contraction, (3) pulsatile activity of oligodendroglia, (4) pinocytosis, (5) mitotic phenomena, (6) activities of the metabolic nucleus, and (7) changes in the juxtanuclear zone.

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Discussion of the Paper

Doctor M. A. Lessler: In relation to the very excellent cinematography of living cells that we have seen I should very much like to hear a discussion of light sources, heating factors, and equipment designed for equal illumination from frame to frame in cine-phase photography of living cells.

Doctor C. M. Pomerat: Illumination must be assessed in cine photography. Even with the use of water filters and a heat-absorbing glass, it is wise to make test runs to evaluate damage in relation to the time of exposure. On one microscope assembly the cells are exposed to light under the desired experimental conditions, while the other microscope unit is run under identical conditions but in darkness. The cells are examined for relative injury at periodic intervals. There is considerable variation in susceptibility to light for various species of cells. The B & L ribbon filament lamp has been found to be ideal for phase-contrast microscopy.

TISSUE CULTURE CADENCES

By Chauncey D. Leake

These notes were inspired during 1953 while watching moving pictures of some of the tissue culture preparations made by Charles M. Pomerat and his associates in the Tissue Culture Laboratory at the University of Texas—Medical Branch in Galveston.

Although first cast in a conventional verse form, the notes were revised, on the advice of competent criticism, into their present less formal and less restrained style. With no pretence at poetical polish, they are merely cadences. They try to express the emotional excitement one may feel at significant new discoveries about living things.

Truth and the Revolving Nucleus

What is the truth
about ourselves and our environment?
How many can agree?
Does each of us
interpret differently
the very same
which all of us can see?

Consider cells,
the unitary base
of every living thing:
so rigid and so set
our concepts of the cell,
by dogma taught,
that biases may bring
the judgments we have sought.

For in the stained and static cell
there is no life;
all motion by the fixative dissolves,
and through the microscope
we see a corpse.
But with contrasting phase,
in time elapsing cinema,
the living cell shows moving parts,
the nucleus revolves!

The Beating Brain

Behold in moving picture all
the marvels of the brain!
It holds such curiosities,
so beauty filled,
with infinite variety of form,
and bubbling, rolling motion which
is never stilled;
with starlike cells,
and neurons reaching far;
with sheaths
pulsating rhythmically, aglim

in constant shift
of swirling, curling, time
elapsing movement on the glowing film.

Is this
the throbbing engine of one's thought,
the intricate
complex machine of mind,
wherein the energy exchanges are
related physically in fact and kind
to symbols which we can communicate?
What greater wonder in
this world to find!

THE SENSITIVE INSIDE OF NOSES

Amusing is the magnified
inside of noses:
here the membraned mucous sheet
is growing, and the cells
in motion swell and veil
their rolling edges, as they beat
the laving fluid into vacuoles.

Look! Slithering, probing blobs
advance, retreat.
Look! In them all
is mitochondric dance,
which slows in cold,
and quick congeals in heat.
Look! On
the glistening surface, cilia wave
like rippling, swaying fields
of wind-blown wheat.

Add pollen extract, or some histamine,
to sensitized mucosa, and it gels
convulsively,
as if it sneezed;
we almost hear
the fevered plaint it tells.

CANCER GROWS WILD

What is the difference in
these lethal shapes,
that makes them deathful deviates
from quiet harmony
with those around
from which they have evolved?
They seem the same,
and yet they are
insidious consumers of
the common nutriment,
harsh foes of ordered life,
cruel tissue tyrants,
whose reeking bloom
all other overgrows.
With steady prying industry,
we find this crablike spread,

called cancer long ago,
may yield the clues
to knowledge and control
of life's evolving mysteries;
our work may show
what cancers are in cells
or in societies:
our efforts may reveal
how life may ebb or flow.

CHANCE AND THE HUMAN CHROMOSOMES

In circled nucleus
the twisted rods lie flat
and silhouette their pregnant shapes
for ease in recognition.
A chance mistake
has pulled away the drapes
of overlapped confusion on
the forms and faces of
the human chromosomes,
so now they may
be named, saluted, while
they stay encased
in their old plasmic homes.

In fantasy we contemplate
the permutations which
there might have been,
had these black rods
but polarized and pulled apart.
We think they may,
fermenting quite unseen,
direct and guide
the symphony of life,
which throbs eternally
in every gene.

EVOLUTION AND THERMODYNAMICS

From all the vast
unbounded sunlit space about
come fragments of an energy
to catalyse our life,
to make the green
of plants,
and in a mystic liturgy
of ordered chance,
to change another gene,
which may produce,
if viable, a kind
of different living thing,
which must, if it survives,
adapt itself to others, as
all of them shall find.

Here in this systemed neat
evolving scheme of everything,
Time's Arrow points the way
inflexibly,

nor can we shift a dot
of its position, nor its movement stay,
nor though we read
the signpost fairly well,
to what it points,
have we the wit to say.

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